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Hypothalamic Pituitary Adrenal Axis Dysregulation in Obese Pregnancy



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A Thesis submitted for the degree of Doctor of Philosophy

School of Clinical Sciences

The University of Edinburgh

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Declaration

I declare that this Thesis has been composed by myself and that the work has not been submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated in the introductory paragraph of each chapter. I confirm that appropriate credit has been given within this Thesis where reference has been made to the work of others.

Laura Ingram Stirrat

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Laura Ingram Stirrat

Edinburgh, 2017

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Abstract and Lay Summary

Abstract

There has been a global rise in obesity in the last three decades, and at present one in five women are obese at antenatal booking. Maternal obesity is associated with an increased risk of adverse pregnancy outcomes, including increased fetal size and prolonged pregnancy. In the longer-term, offspring of obese are at increased risk of premature death from a cardiovascular event in their adulthood. One mechanism that has been linked to these outcomes is fetal exposure to glucocorticoids *in utero*.

During normal pregnancy, the maternal hypothalamic pituitary adrenal (HPA) axis undergoes major changes, resulting in exponentially increasing levels of the major circulating glucocorticoid cortisol, and other HPA axis hormones, such as corticotrophin releasing hormone (CRH). Cortisol and CRH are vital for normal fetal growth and length of gestation, but in excess they are associated with fetal growth restriction and preterm labour.

In non-pregnant obesity, it is thought that the HPA axis is dysregulated, although evidence is inconclusive. Little is known about the effects of maternal obesity in pregnancy on the HPA axis. The work in this Thesis used clinical studies to test the hypothesis that the HPA axis is dysregulated in obese pregnant women with altered release, clearance and placental metabolism of cortisol. Associations with clinical outcomes related to fetal size and length of gestation were also studied.

The HPA axis activity during pregnancy was investigated in a prospective case-control study cohort. Fasting serum cortisol levels were measured at 16, 28 and 36 weeks of gestation (obese n=276, lean n=135). In a subset (obese n=20, lean n=20), corticosteroid binding globulin (CBG), CRH, estrogens and progesterone were measured. Salivary cortisol was measured in samples collected at bedtime,

waking and 30 minutes after waking at 16 weeks. Urinary glucocorticoid metabolites were measured at 19 weeks and 36 weeks (obese n=6, lean n=5) and non-pregnant (obese n=7, lean n=7) subjects. All circulating hormone levels rose similarly in obese and lean during pregnancy, but were significantly lower in obese women. The diurnal rhythm of cortisol was maintained. Urinary glucocorticoids increased with gestation in lean, but not in obese, indicating a lesser activation of the HPA axis in obese compared with lean pregnancy. These findings associated with increased birthweight and longer gestation in obese pregnancy, suggesting that decreased HPA axis activity may underlie these obese related adverse pregnancy outcomes.

Whether or not lower glucocorticoids in obese pregnancies are maintained at delivery was investigated by measuring active glucocorticoids (cortisol and corticosterone) and their inactive versions (cortisone and 11-dehydrocorticosterone, respectively) from matched maternal and cord plasma samples (n=259, BMI 18 – 55 kg/m²). Active glucocorticoids were significantly higher in maternal than cord blood, and inactive versions were significantly higher in cord than maternal blood. Increased maternal BMI associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone. Despite significant correlations between maternal and cord blood glucocorticoid levels, increased maternal BMI did not associate with lower cord blood glucocorticoids. This suggests that conditions at delivery may overcome any potential negative effects of low maternal glucocorticoids on the fetus in the short-term. However, it may not preclude the longer-term effects of fetal exposure to lower glucocorticoid levels during obese pregnancy, and offspring follow-up studies are required.

Potential mechanisms leading to altered HPA axis activity in obese pregnancy were explored by studying the pulsatile release and placental metabolism of glucocorticoid hormones. Glucocorticoid pulsatility is thought to be important for transcriptional regulation of glucocorticoid responsive genes, and disruptions

to pulsatility have been reported in some disease processes. Glucocorticoids were measured in 10-minute serum sampling between 08.00h-11.00h and 16.00h-19.00h. Peripheral tissue cortisol was measured from 20-minute sampling of interstitial fluid, over 24-hours, at 16-24 weeks and 30-36 weeks (obese n=7, lean n=8), and non-pregnant controls (obese n=4, lean n=3). Total circulating serum cortisol levels were higher in pregnancy than non-pregnancy in lean and obese, and increased significantly with advancing gestation in lean but not in obese. Pulsatility of cortisol was demonstrated in interstitial fluid in both non-pregnancy and pregnancy. In obese pregnancy, interstitial fluid pulse frequency was lower with advancing gestation. This may be a novel mechanism underlying the observed decreased HPA axis activity in obese pregnancy.

Placental cortisol metabolism and transport was studied using an *ex vivo* placental perfusion model, perfused with a deuterium-labelled cortisol tracer combined with computational modeling. The findings challenge the concept that maternal cortisol diffuses freely across the placenta, but confirmed that 11 β -HSD2 acts as major 'barrier' to cortisol transfer to the fetus, protecting the fetus from the high maternal circulating cortisol levels. In addition we showed preliminary evidence of local cortisol production within the placenta. The model is able to predict maternal-fetal cortisol transfer and can now be used in future experimental design.

In conclusion, in obese pregnancy, lower maternal cortisol and urinary clearance suggested reduced HPA axis activity. Altered glucocorticoid pulsatility may underlie this change. Future studies of placental cortisol metabolism in maternal obesity could be conducted using an *ex vivo* perfusion model. The lower HPA axis activity in obese pregnancy represents a novel pathway underlying increased fetal growth.

Lay Summary

There has been a global rise in obesity in the last three decades, and one in five women are obese at the time of their first antenatal appointment. Obesity during pregnancy is associated with increased birthweight of the baby, and a higher chance that the pregnancy will continue beyond the estimated due date. Offspring of women who were obese during pregnancy are also more likely to die at a younger age in their adulthood, as a result of heart disease. One mechanism that has been linked to these outcomes is exposure of the developing baby to 'stress hormones' during pregnancy.

During normal pregnancy, the 'hypothalamic pituitary adrenal axis', which is a family of naturally occurring 'stress hormones' undergoes major changes, and the result of this is that the levels of stress hormones such as cortisol are much higher. Cortisol is important for normal growth of the baby during pregnancy, and for the length of the pregnancy. However, in pregnancies where cortisol levels are abnormally high, the overall growth of the baby can be restricted, and labour may occur prematurely.

In non-pregnant obese women, it is thought that cortisol levels become imbalanced. However, it is not known whether cortisol levels are affected by obesity during pregnancy. The work in this thesis aimed to study whether or not cortisol levels are altered in obese pregnancy, and to identify potential reasons for any alterations. These included studying the release (in blood) and clearance (in urine) of cortisol, and studying how the placenta processes cortisol.

Cortisol measured from blood samples from 276 obese and 135 normal weight women at 16, 28 and 36 weeks of pregnancy, showed that levels increased as pregnancy progressed, and were lower in obese than normal weight pregnant women. Urine samples showed that the breakdown products of cortisol increased as pregnancy progressed in normal weight women, but not in obese women. This

suggested that the lower levels of cortisol in blood could not be explained by higher breakdown and clearance in the urine. These results were also linked to increased offspring birthweight and longer length of pregnancy, suggesting that lower levels of cortisol in obese pregnant women may be linked to these pregnancy outcomes.

Blood samples obtained from pregnant women and their babies (via the umbilical cord) at the time of delivery, showed that cortisol levels were lower in obese than normal weight women, but there were no differences in the samples collected from the umbilical cord. This suggests that the 'stressful' conditions at delivery may overcome any potential effect of the mother being obese at the time of delivery. However, it may not negate the longer-term effects of the developing baby being exposed to lower levels of stress hormones during pregnancy, so longer-term studies of the offspring are required.

Cortisol is released in pulses, and these pulses are important for maintaining normal genetic processes. In order to investigate the effect of obesity on the pulsatile release of cortisol, frequent samples of blood and peripheral tissue stress hormone levels were collected at 16-24 weeks and 30-36 weeks of pregnancy, in obese and lean pregnant, and non-pregnant women. Pulses were detected in the peripheral tissue fluid and occurred less frequently in obese than normal weight pregnant women. Less frequently occurring cortisol pulses may be an underlying reason for the lower levels of stress hormones demonstrated in obese pregnant women. The way the placenta processes cortisol was investigated by pumping solutions with different concentrations of cortisol through a piece of placenta, within 30-minutes of delivery of the baby. The results challenged a previously held thought that cortisol can pass freely through the placenta, and confirmed that an enzyme within the placenta acts as a major 'barrier' to prevent the developing baby from being exposed to abnormally high levels of cortisol. These experiments also showed preliminary evidence that the placenta can produce cortisol. This model could be used for future experiments to study

cortisol transport between the mother and baby and how the placenta processes cortisol. In conclusion, in obese pregnant women, lower levels of cortisol in blood and urine suggest that the activity of stress hormones are reduced compared with normal weight pregnant women. An altered pattern of cortisol pulses may underlie this change. Future studies of the way the placenta processes cortisol could be conducted using the experiments we used. Lower activity of stress hormones in obese pregnancy represents a novel underlying pathway for increased baby birthweight in babies of obese mothers.

Publications and Presentations

Relating to this Thesis

Poster Presentations

- **Hypothalamic Pituitary Adrenal Axis Dysregulation in Obese Pregnancy: Clinical Implications and Underlying Mechanisms.**

Laura Stirrat, Ksenia Stryjakowska, Sarah Barr, Ruth Andrew, Stafford Lightman, Jane Norman, Rebecca Reynolds.

Society for Reproductive Investigation: Targeting Inflammation, 2017, Edinburgh, UK.

- **Glucocorticoids are lower at delivery in maternal, but not cord blood of obese pregnancies.**

Laura Stirrat, George Just, Natalie Homer, Ruth Andrew, Jane Norman and Rebecca Reynolds

British Maternal and Fetal Medicine Society Annual Meeting, 2017, Amsterdam, The Netherlands.

Shortlisted for the 'Best Poster' prize

- **Cortisol Pulsatility in Pregnancy: a case-control study.**

Laura Stirrat, Jamie Walker, Jane Norman, Stafford Lightman and Rebecca Reynolds.

Academy of Medical Sciences Spring Meeting, 2016, London, UK.

- **Altered maternal hypothalamic-pituitary-adrenal axis activity in obese pregnancy: a potential mechanism underlying macrosomia and prolonged pregnancy.**

Laura Stirrat, James O'Reilly, Alexander Howie, Roger Smith, Brian Walker, Jane Norman, Rebecca Reynolds.

British Maternal and Fetal Medicine Society, 2014, Harrogate, UK.

Oral Presentations

- **Transfer and Metabolism of Cortisol by the Isolated Perfused Human Placenta**

Laura Stirrat, Bram Sengers, Rohan Lewis, Rebecca Reynolds

European Placental Perfusion Network, 2017, Geneva, Switzerland.

- **Cortisol pulsatility is reduced and may underlie decreased hypothalamic-pituitary-adrenal-axis activity in obese pregnancy.**

Laura Stirrat, Ksenia Stryjakowska, Natalie Jones, George Just, Natalie Homer, Jamie Walker, Stafford Lightman, Ruth Andrew, Jane Norman and Rebecca Reynolds.

British Maternal and Fetal Medicine Society Annual Meeting, 2017, Amsterdam, The Netherlands.

- **Reduced cortisol pulsatility and cortisol placental metabolism may underlie decreased hypothalamic-pituitary-adrenal-axis activity in obese pregnancy.**

Laura Stirrat, Ksenia Stryjakowska, Sarah Barr, Ruth Andrew, Stafford Lightman, Jane Norman, Rebecca Reynolds.

Royal College of Obstetricians and Gynaecologists Annual Academic Meeting, 2017, London, UK.

- **Reduced cortisol pulsatility and cortisol placental metabolism may underlie decreased hypothalamic-pituitary-adrenal-axis activity in obese pregnancy.**

Laura Stirrat, Ksenia Stryjakowska, Sarah Barr, Ruth Andrew, Stafford Lightman, Jane Norman, Rebecca Reynolds.

Edinburgh Obstetrical Society Juniors', 2016, Edinburgh, UK. Presented as an oral presentation.

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- **Hypothalamic Pituitary Adrenal Axis Hormones Are Reduced In Obese Pregnancy.**

Laura Stirrat, James O'Reilly, Simon Riley, Alexander Howie, Maria Bowman, Roger Smith, John Lewis, Brian Walker, Jane Norman, Rebecca Reynolds.

Society for Reproductive Investigation, 2015, San Francisco, California, USA.

Awarded the '2015 President's Presenter Award'

- **Hypothalamic pituitary adrenal axis dysregulation in obese pregnancy may influence fetal growth and length of gestation.**

Laura Stirrat, James O'Reilly, Rebecca Reynolds

Association for the Study of Obesity Conference, 2014, London, UK.

Publications

- Stirrat LI, Sengers BG, Norman JE, Homer NZM, Andrew R, Lewis RM, Reynolds RM. Transfer and Metabolism of Cortisol by the Isolated Perfused Human Placenta. *The Journal of Clinical Endocrinology and Metabolism*. 2017 Nov 16; doi: 10.1210/jc.2017-02140. [Epub ahead of print].
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term outcomes for offspring. *Research and Reports in Neonatology*. 2014 Mar;4:43-53.

Abbreviations

Abbreviation	Full Definition
11 β -HSD1	11-beta-hydroxysteroid dehydrogenase type 1
11 β -HSD2	11-beta-hydroxysteroid dehydrogenase type 2
ABC-Transporters	ATP-Binding Cassette
ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
	Altered Metabolic Processes in Obese Pregnant Women - Clinical study
AMPOP	
ANOVA	Analysis of variance
AUC	Area under the curve
BCRP	Breast-cancer-resistant protein
BM	Basement membrane
BMI	Body mass index
BSA	Bovine serum albumin
Cacl2	Calcium chloride
CBG	Corticosteroid binding globulin
CBX	Carbenoxolone
CMACE	Centre for Maternal and Child Enquiries
CO2	Carbon dioxide
CRH	Corticotrophin releasing hormone
CRHBP	Corticotrophin releasing hormone binding protein
CV	Coefficient of Variation
D3E	D3-Cortisone
D3F	D3-Cortisol
D4F	D4-Cortisol
Da	Daltons
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
E2	Estradiol
E3	Estriol
EBB	Earle's bicarbonate buffer
EDTA	Ethylenediaminetetraacetic acid
EICS	Elective Caesarean Section
ELISA	Enzyme linked immunoassay
EmCS	Emergency Caesarean Section
	Effect of metformin on maternal and fetal outcomes in obese pregnant women study
EMPOWaR	
Eq	Equation

ERTBB	Edinburgh Reproductive Tissue BioBank
ERTBB	Edinburgh Reproductive Tissue BioBank
GDM	Gestational diabetes
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
h	Hours
HIP	Hormones in Pregnancy
HPA	Hypothalamic pituitary adrenal
HSIC	Health and Social Care Information Centre
ISD	Information Services Division
k_{BM}	BM permeability constant
KCl	Potassium chloride
K_m	Michaelis-Menton constant
k_{MVM}	MVM permeability constant
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LGA	large for gestational age
LNP	Lean non pregnant
LOQ	Limit of quantification
LP1	Lean pregnant visit 1
LP2	Lean pregnant visit 2
MAC	Metabolic Antenatal Clinic
MBRACE-UK	Mother's and Babies: Reducing Risk through Audits and Confidential Enquiries in the UK
MDC	Minimum detectable concentration
MgSO ₄	Magnesium sulphate
mmHg	Millimetre of mercury
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MRP1	Multi drug resistant protein 1
MRP1	multi-drug-resistant protein
MS/MS	Tandem mass spectrometry
MVM	Microvillous membrane
N ₂	Nitrogen
NaCl	Sodium chloride
NaH ₂ PO ₄ .2H ₂ O	Sodium phosphate dibasic dihydrate
NaHCO ₃	Sodium bicarbonate
NHS	National Health Service
ONP	Obese non pregnant
OP1	Obese pregnant visit 1

OP2	Obese pregnant visit 2
P-gp	P-glycoprotein
P4	Progesterone
PBS-T	Phosphate buffered saline with tween
<i>Per1</i>	Period circadian protein homolog
PIPa	The Effect of Maternal Weight and Gestation on Cortisol Pulsatility in Pregnancy - Clinical study
Q_f	Fetal flow rate, L/min
Q_m	Maternal flow rate, L/min
Qual	Qualifier ion
Quan	Quantifier ion
RDS	Respiratory distress syndrome
REC	Research Ethics Committee
RIA	Radioimmunoassay
RSD	Relative standard deviation
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SD	Standard Deviation
SDS	Standard Deviation Score
SEM	Standard error of the mean
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin
SLE	Supported liquid extraction
SNAT2	System A Amino Acid Transporter 2
SPD	Symphysis pubis dysfunction
StAR	Steroidogenic acute regulatory protein
SVD	Spontaneous Vaginal Delivery
THE	Tetrahydrocorticone
THF	Tethydrocortisol
THF	5 β -tetrahydrocortisol
TMB	3,3',5,5'-tetramethylbenzidine
UFC	Urinary free cortisol
UK	United Kingdom
UPBEAT	UK Pregnancies: Better Eating and Activity Trial
UPLC	Ultra performance liquid chromatography
USA	United States of America
V	Volts
V_f	Fetal compartment volume
V_m	Maternal compartment volume
V^{\max}	Maximum rate of reaction
V_s	Syncytiotrophoblast compartment volume

WHO	World Health Organisation
WHR	Waist hope ratio
α -THF	5 α -tetrahydrocortisol

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Chapter 1

Introduction

1.1 Obesity in Pregnancy

1.1.1 Global Incidence

Obesity is defined as ‘abnormal or excessive fat accumulation that may impair health’ by the World Health Organisation (WHO). Body mass index (BMI) is a crude population measure of obesity that is an index of weight-for-height. In adults, overweight is defined as a BMI of $\geq 25 \text{ kg/m}^2$, and obesity is defined as a BMI $\geq 30 \text{ kg/m}^2$.

During the last three decades there has been a global rise in the incidence of obesity. The number of overweight and obese individuals worldwide increased from 857 million in 1980, to 2.1 billion in 2013 (Ng et al., 2014). Between 1975 and 2014, the age-standardised prevalence of obesity in men increased from 3.2% to 10.8%, and increased from 6.4% to 14.9% in women (Risk Factor Collaboration, 2016). If these trends continue, it is estimated that by 2025, the global obesity prevalence will be 18% in men and 21% in women (Risk Factor Collaboration, 2016). In 2015 the Scottish Health Survey reported that 65% adults aged 16-64 were overweight, and 29% were obese (Rose, 2015).

As the prevalence of obesity increases, so does the number of women of reproductive age who are becoming overweight and obese (Linne, 2004). In 2009 maternal obesity was reported to affect at least 20% of pregnancies in the United States (Chu et al., 2009). In 2010 a national audit of obesity during pregnancy was conducted across the UK by the Centre for Maternal and Child Enquiries (CMACE) from 1 March – 30 April 2009; this reported that at least 5% of pregnant women in the UK are severely obese (BMI $> 35 \text{ kg/m}^2$) (CMACE,

2010). Recent national statistics from the UK suggest that similar rates of maternal obesity are persisting. The Health and Social Care Information Centre (HSIC) reviewed data from the first antenatal appointment of 33,950 pregnancies in England in September 2015, and reported that 23% were obese at antenatal booking (HSIC, 2015). In Scotland, the Information Services Division (ISD) reported that 22.2% of women delivering in Scottish hospitals in 2016 were obese at the time of antenatal booking (ISD, 2016). This significantly high proportion of women who are obese means there is an equally high proportion of offspring who have been exposed to the obesogenic environment *in utero*.

1.1.2 Risks of Maternal Obesity in Pregnancy

Maternal obesity is associated with an increased risk of adverse outcomes for both the mother and the baby, including maternal, fetal and neonatal mortality. In the most recent confidential enquiry into maternal deaths in the United Kingdom (MBRACE-UK, 2016), 65 women (33%) who died had a BMI > 30 kg/m². Adverse pregnancy outcomes and short-term neonatal complications associated with maternal obesity have been well described (Galliano and Bellver, 2013, Vasudevan et al., 2011, Sebire et al., 2001, Dinatale et al., 2010) and it is recognised that obese pregnant women should receive consultant-led, multi-disciplinary care (Ghaffari et al., 2015). A recent study examining pregnancy outcomes of obese women in NHS Lothian found that women who attended a specialist high-risk clinic for women with BMI > 40 kg/m², were less likely to have a stillbirth or a low birthweight baby (Denison et al., 2017)

Obese women are more likely to experience infertility (Mitchell and Fantasia, 2016) and those who do become pregnant are at increased risk of miscarriage from both spontaneous (Boots and Stephenson, 2011) and assisted conception (Metwally et al., 2008). Obesity has also been identified as an independent risk

factor for recurrent, unexplained miscarriage (Lashen et al., 2004), with up to 73% increased risk of another miscarriage (Lo et al., 2012).

Obese women are at increased risk of major antenatal complications, including gestational diabetes (Leddy et al., 2008, Denison et al., 2014), gestational hypertensive disorders (Leddy et al., 2008, Denison et al., 2014), and thromboembolic disease (Larsen et al., 2007, Jacobsen et al., 2008). Minor complications are also more prevalent during pregnancy in the obese population. These include respiratory tract and chest infections, gastro-oesophageal reflux, carpal tunnel syndrome and symphysis pubis dysfunction (SPD) (Denison et al., 2009).

Later in pregnancy, obese women are at increased risk of having a prolonged or post-dates pregnancy (Denison et al., 2008, Heslehurst et al., 2017), which increases the need for induction of labour and increases the risk of stillbirth (Flenady et al., 2011, Leddy et al., 2008). Operative or instrumental deliveries are more prevalent in obese women (Hollowell et al., 2013). The risk of Caesarean section due to previous Caesarean delivery, failure to progress in labour, failed induction of labour or fetal distress is higher in obese compared with normal weight women (Fernandez Alba et al., 2017), and the risk increases progressively with increasing maternal BMI. Obese patients undergoing Caesarean delivery have an increased risk of surgical complications, including wound dehiscence, infectious morbidity and hospital readmission (Owen and Andrews, 1994, Vermillion et al., 2000, Stamilio and Scifres, 2014). Maternal obesity may reduce surgical exposure, increase the duration of operation, increase blood loss and increase length of hospitalisation (Perlow and Morgan, 1994).

1.1.3 Short-Term Offspring Outcomes

Offspring of obese women are at increased risk of congenital malformations (Stothard et al., 2009). Potential mechanisms may include deficiencies in nutrients such as folic acid, chronic hypoxia, and metabolic changes including maternal hyperglycaemia, increased insulin resistance, and increased circulating levels of triglycerides and uric acid (Stirrat and Reynolds, 2014). Relative difficulties with antenatal detection may also contribute to the apparent increase in congenital abnormalities. Ultrasound scanning of obese pregnant women may lead to suboptimal visualisation of fetal anatomy (Hendler et al., 2005), lower detection rates of structural abnormalities, and therefore an increased prevalence at birth.

Maternal obesity is associated with up to a 72% increased risk of having a large for gestational age (LGA) baby, independent of co-existent diabetes (Ehrenberg et al., 2004). In addition to being born LGA, anthropometric measurements of neonates born to obese women demonstrate they are more likely to have a significantly higher fat mass and a higher percentage of body fat (Sewell et al., 2006). LGA infants are predisposed to a variety of obstetric and neonatal complications due to potential difficulties during labour and delivery, such as shoulder dystocia and brachial plexus injury (Walsh et al., 2011).

In the neonatal period, macrosomic infants (often defined as birthweight ≥ 4000 g at ≥ 37 weeks gestation) are more likely to have electrolyte and metabolic disturbances, such as hypoglycaemia, hyperbilirubinemia and hypomagnesemia (Nold and Georgieff, 2004). In the long-term, infants that are at the highest end of the distribution for weight or BMI are at greater risk of being obese in childhood, adolescence, and early adulthood (Boney et al., 2005), and are at risk of cardiovascular and metabolic complications later in life (Hermann et al., 2010, Ornoy, 2011). Potentially modifiable predictors of birthweight include maternal weight, gestational weight gain, gestational age at birth, and maternal glucose

metabolism. An interesting paradox exists, with some reports suggesting that offspring of obese women are also at increased risk of intrauterine growth restriction (unrelated to pre-eclampsia) (Rajasingam et al., 2009). Mechanisms underlying these findings are not known but may be related to poor placental function.

Babies of obese mothers have up to a 31% increased risk of have a low Apgar score at birth (defined as Agar Score < 7 at 1 minute) (Kalk et al., 2009). Offspring from overweight or obese mothers appear to be at up to 38% increased risk of being admitted to the neonatal unit (Kinnunen et al.) at birth than offspring of mothers with a normal BMI (Minsart et al., 2013). Higher rates of admission for treatment of neonatal hypoglycaemia have been reported in offspring from obese mothers (Kalk et al., 2009).

1.1.4 Long-Term Offspring Outcomes

The ‘Barker hypothesis’ states that environmental influences acting in fetal life are reflected in impaired growth and developments which permanently affect structure and metabolism, leading to increased risk of metabolic disease later in life (Barker, 1998). This theory stimulated interest in the fetal origins of adult disorders, which then expanded and coalesced with the formation of an international society for the Developmental Origins of Health and Disease (DOHaD). The original Barker hypothesis has mainly focussed on under-nutrition in the pregnant mother. However, during obese pregnancy, it has been suggested that the increased nutrient supply may lead to a response by the fetus to ‘program’ its organs and tissues in a way that translates to long-term alterations to their function (Lucas, 1991). This has been termed the ‘developmental over-nutrition’ hypothesis (Taylor and Poston, 2007). There is a

growing body of evidence that there is a long-term relationship between maternal obesity and cardio-metabolic health in adulthood for the offspring.

Offspring of obese have an increased risk of both childhood and adulthood obesity (Drake and Reynolds, 2010, Catalano et al., 2009), and this appears to be independent of maternal diabetes (Sewell et al., 2006). Both macrosomia and excessive gestational weight gain during pregnancy are strong predictors of higher BMI at one year of age, and BMI at one year of age is predictive of weight at five-eight years of age (Lindberg et al., 2012). A sibling study using a cohort of mothers who underwent surgical interventions for obesity found that offspring born before surgical intervention had significantly higher bodyweights at 12 years and at 21-25 years, than offspring born after surgery (Barisione et al., 2012).

There is increasing evidence to support a link between maternal obesity, offspring obesity and cardiometabolic risk factors (Drake and Reynolds, 2010). Maternal obesity has been associated with offspring insulin resistance in newborn babies (Catalano et al., 2009), at 11 years (Boney et al., 2005) and at age 20 years (Mingrone et al., 2008). Maternal obesity has been shown to be independently correlated with higher systolic and diastolic blood pressure in offspring at 17 years (Laor et al., 1997) and at age 32 years (Hochner et al., 2012). In the same cohort, maternal BMI was also significantly associated with lower high density lipoproteins at age 32 years (Hochner et al., 2012). These associations were significant when separated from 'shared environment' and 'postnatal lifestyle' factors. This would support a programming influence of maternal obesity of offspring obesity.

Recent evidence has suggested that these associations may translate into increased risk of cardiovascular disease and death for the offspring. A study investigating the relationship between maternal obesity and premature adults mortality in 37,709 offspring from obese mothers (Reynolds et al., 2013), found

offspring of obese mothers had a 40% increased risk of premature death from all causes, and at 29% increased risk of death due to cardiovascular disease. The risk was independent of socioeconomic status. Only 4% of mothers in this study were obese, far fewer than current obesity levels, thus these findings are alarming.

Offspring of obese women are also thought to be at increased risk of asthma (Harpsoe et al., 2013), neurodevelopmental disorders such as autism spectrum disorders and developmental delay (Krakowiak et al., 2012). Observational evidence suggests that increased maternal BMI is an independent risk factor for schizophrenia in offspring, when controlling for other potentially confounding maternal characteristics (Schaefer et al., 2000).

The effects of the positive feedback loop of adiposity from obese mothers to the child may increase the risk of some cancers for the offspring (Simmen and Simmen, 2011) with which birth weight is positively associated (Andersson et al., 2001, Ahlgren et al., 2007).

1.2 The Hypothalamic Pituitary Adrenal Axis

1.2.1 Glucocorticoids

Glucocorticoids are vital for life, and are involved in regulating or supporting cardiovascular, metabolic, immune, and homeostatic functions. Cortisol is the major circulating glucocorticoid hormone in humans. There is also an increasing interest in the potential physiological roles of glucocorticoid hormone corticosterone, which comprises 5-10% of total plasma glucocorticoids (Nixon et al., 2016). These glucocorticoid hormones are synthesised in the zona fasciculata of the adrenal cortex, under the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1-1**). Glucocorticoid synthesis is initiated by transport of cholesterol into the inner mitochondrial membrane by the steroidogenic acute

regulatory protein (StAR) (Stocco and Clark, 1996). Following a series of enzyme catalysed conversion steps, glucocorticoids are formed (Bush, 1953, Hum and Miller, 1993). Secretion of glucocorticoids is regulated by adrenocorticotrophic hormone (ACTH), secreted by the pituitary gland, and ACTH is under the control of corticotrophin releasing hormone (CRH), secreted from the paraventricular nucleus of the hypothalamus (Vale et al., 1981). The HPA axis is a series of feedback interactions between the paraventricular nucleus of the hypothalamus, the anterior pituitary gland and the adrenal cortex. CRH is released from the hypothalamus in response to stress, illness, physical activity, levels of circulating cortisol in the bloodstream, and also by the sleep-wake 'circadian rhythm' cycle.

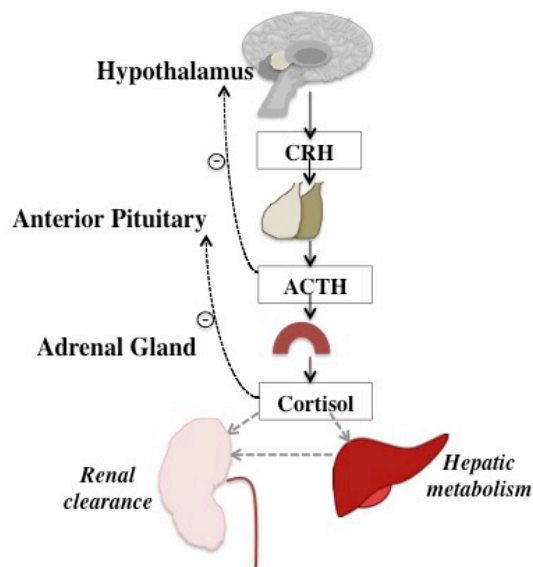


Figure 1-1. The Hypothalamic Pituitary Adrenal Axis in Humans

Glucocorticoids bind to glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) (Bamberger et al., 1996). These receptors are activated upon ligand binding and the receptor-ligand complex translocates to the nucleus, where it binds to glucocorticoid response elements in the promoter region of target genes, and can then influence gene transcription. Glucocorticoids can also exert non-genomic effects by direct actions on membrane lipid and cytoplasmic proteins via membrane-located receptors (Haller et al., 2008). GR is expressed from mid-gestation onwards in fetal tissues, the placenta and fetal membranes (Cole et al., 1995); and animal studies suggest that MR is more limited and tends to be present later in gestation (Brown et al., 1996b).

The majority of cortisol metabolism takes place in the liver by A-ring reductase enzymes. Additional metabolism occurs by enzymes 5 α -reductase in fat, and by 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) in the distal nephron of the kidney (Chapman et al., 2013), which converts active cortisol into cortisone (its inactive version). Cortisol can also be regenerated from cortisone by enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1). The extent of regeneration of cortisol from cortisone by 11 β -HSD1 in liver or fat influences the metabolic clearance rate (Phillipov et al., 1996). Free cortisol and metabolites of cortisol (glucocorticoid metabolites; **Figure 1-2**) are excreted in urine via the renal system.

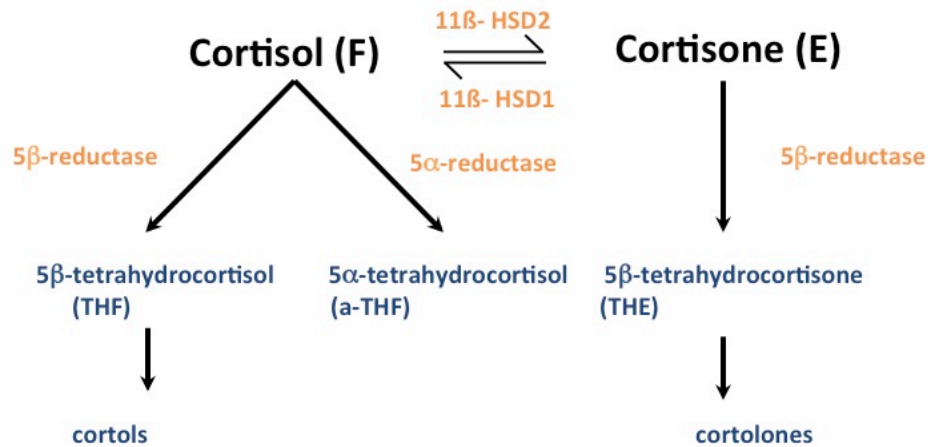


Figure 1-2. The Metabolism of Cortisol by 11beta-hydroxysteroid dehydrogenase (11β-HSD2) and 5α- and 5β-reductase.

Key: Tetrahydrocortisol (THF), tetrahydrocortisone (THE).

1.2.1.1 Circadian Rhythm of Cortisol

Circadian rhythms are 24-hour cycles in bodily processes that allow organisms to anticipate and prepare for changing environmental factors such as light and dark or seasonal changes. Such rhythms exist in all organisms, and can affect biochemistry, physiology, endocrinology and behavioural (Panda et al., 2002).

The HPA axis is under circadian regulation, which in humans is characterised by peak levels of cortisol early in the morning at the end of the ‘resting’ overnight period, in preparation for the increased metabolic demands of the active day time phase (Young et al., 2004). Nocturnal animals such as rodents exhibit a peak in corticosterone levels (corticosterone is the major circulating glucocorticoid in rodents) towards the end of the afternoon, when the dark cycle begins (Gong et al., 2015). This daily rhythm arises from a genetically operated ‘biological clock’ (Dunlap, 1999, Reppert, 1998). In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus functions as the master circadian pacemaker, driving the rhythmic secretion of hormones (Stratmann and Schibler, 2006). Daily cyclic variations in glucocorticoid hormone concentrations are thought to be fundamental for the overall maintenance of physiology and well being (Young et al., 2004, Lightman and Conway-Campbell, 2010). The circadian rhythm of the HPA axis develops sometime after birth and can be detected as early as one month of age in humans (Ivars et al., 2015).

1.2.1.2 Ultradian Rhythm of Cortisol

Knowledge and understanding of basal circadian rhythmicity has been advanced with the development of automated sampling devices (Jasper and Engeland, 1991, Windle et al., 1998b, Tapp et al., 1984, Cook, 2001, Weitzman et al., 1971, Henley et al., 2009a). These facilitate frequent sampling without repeated insertion of needles, which would mount a stress response. Furthermore, the development of intra-tissue microdialysis sampling for use in animals (Droste et al., 2008) and humans (Bhake et al., 2013), and mathematical algorithms for analysis (Veldhuis et al., 1989, Merriam and Wachter, 1982), which accounts for secretory dynamics, have facilitated a more in depth study of HPA axis activity. Together these advances have contributed to the discovery that underlying the circadian release of glucocorticoids are discrete pulses of HPA axis hormones,

forming a rapid and dynamic ‘ultradian rhythm’ (Veldhuis et al., 1989). This is a recurrent cycle of hormone oscillations in blood and target tissues occurring approximately hourly (Veldhuis et al., 1989, Follenius et al., 1987, Jasper and Engeland, 1991), repeated throughout a 24-hour circadian day (Spiga et al., 2014). This type of pattern was previously interpreted as ‘noise’ in the system, because it was particularly difficult to study basal, unstressed conditions. The ‘amplitude’ and ‘frequency’ of these oscillations, or pulses, increase during the circadian peak of secretion (Veldhuis et al., 1989). Intracranial microdialysis studies in animals have demonstrated that ultradian pulses of corticosterone are synchronised in plasma and in the brain (Droste et al., 2008). The pulsatile pattern of glucocorticoids has been described in a number of species. These include rat (Atkinson et al., 2006, Jasper and Engeland, 1991, Windle et al., 1998a), rhesus monkey (Sarnyai et al., 1995, Tapp et al., 1984), Syrian hamster (Loudon et al., 1994, Lucas et al., 1999), horse (Cudd et al., 1995), sheep (Engler et al., 1989), goat (Carnes et al., 1992) and humans (Hellman et al., 1970, Veldhuis et al., 1990, Weitzman et al., 1971).

Until recently, it had been assumed that glucocorticoid pulses originated from a ‘pulse generator’ such as the SCN (Ixart et al., 1991). This theory worked on the assumption that the pulsatile characteristics of CRH (Ixart et al., 1991, Caraty et al., 1988, Ixart et al., 1993) would drive ACTH release (Carnes et al., 1988), that would in turn cause glucocorticoid pulsatility, thus resulting in the ultradian rhythm. However, studies in rodents have revealed a mismatch in pulse frequency; CRH was found to have three pulses per hour (Ixart et al., 1991) compared to hourly pulses of ACTH (Carnes et al., 1986) and corticosterone (Windle et al., 1998b). In sheep, the pulsatility of both ACTH and glucocorticoids have been shown to continue even after disconnection of the hypothalamus from the pituitary gland (Engler et al., 1990), which supports a sub hypothalamic origin. Further still, in SCN-lesioned rodents, the ultradian rhythm

of corticosterone has been shown to persist despite a loss in circadian rhythmicity (Waite et al., 2012).

Walker et al., (Walker et al., 2012) demonstrated that pulsatile release of CRH is not needed for ultradian of the pituitary-adrenal system to continue, and supported that hypothesis that glucocorticoid inhibition at the anterior pituitary plays a key role in regulating the ultradian rhythm of glucocorticoid release. Their model of sub-hypothalamic origin of pituitary-adrenal ultradian pulses was tested *in vivo* by measuring ACTH and corticosterone in freely behaving rats under different rates of constant CRH infusions at the circadian trough when endogenous CRH drive was very low and there was minimal secretion of endogenous ACTH and corticosterone. As predicted by a mathematical model (Walker et al., 2010), the constant infusion of CRH did induce ultradian release of both ACTH and corticosterone with consistent pulse amplitude and frequency for the duration of the infusion, and the ACTH pulses preceding corticosterone pulses. High levels of CRH infusion disrupted the pattern leading to large pulses of ACTH and corticosterone, followed by a subsequent dampening of corticosterone pulses. These data confirmed that ultradian oscillations of the pituitary-adrenal system are a result of the feedforward:feedback relationship of ACTH with the adrenal cortex and endogenous glucocorticoids on the pituitary corticotrophs (Walker et al., 2010, Walker et al., 2012). Whether pulsatility of hypothalamic CRH secretion could impact on this sub-hypothalamic system is as yet unknown.

As previously described (Droste et al., 2008), ultradian glucocorticoid pulses in plasma are paralleled in brain tissue. This would suggest that the local neuronal target environments where GR and MR are found, are not in constant equilibrium. On the contrary, it would appear that they are exposed to a rapidly changing concentration of their ligand, glucocorticoids (de Kloet and Sarabdjitsingh, 2008). This has been confirmed in both *in vitro* and *in vivo* animal models, where it has been shown that pulses of tissue corticosterone are

paralleled by pulses of GR binding and gene transcription. Studies in cultured cells and in animal models have confirmed that oscillations in glucocorticoids induce a phenomenon known as ‘gene pulsing’ (Stavreva et al., 2009, Conway-Campbell et al., 2010). During periods of low hormone concentration such as during the nadir of the circadian rhythm, GR is thought to be present in the cytoplasmic compartment where ligand binding occurs. When glucocorticoid levels rise during an endogenous pulse, glucocorticoids bind to and activate GR, which then translocate into the cell nucleus. After interacting with glucocorticoid response elements (GRE), the complex binds to DNA to initiate transcription.

After withdrawal of the ligand, GR has been shown to remain in the nucleus for several hours (Stavreva et al., 2015, Stavreva et al., 2004, Yang et al., 1997, Carrigan et al., 2007), and while unliganded, GR does not associate with chromatin (Stavreva et al., 2009). After a fresh pulse of glucocorticoid, there was a re-activation of GR within the nuclear compartment and re-association of GR with the chromatin template (Stavreva et al., 2009).

In contrast to pulsatile presentation of glucocorticoids and pulsatile response of gene transcription, constant presentation of glucocorticoids is associated with a change in genomic responses in a brain-area and cell-type specific fashion (Conway-Campbell et al., 2010, Stavreva et al., 2015), where transcription levels of many glucocorticoid responsive genes continue to rise (Stavreva et al., 2009). This suggests that it is ultradian rhythm rather than the absolute level of glucocorticoids present that are necessary to maintain transcriptional efficiency in target tissues (Stavreva et al., 2009). Interestingly, only endogenous (as opposed to exogenous) glucocorticoids led to the pulsatile GR activation profiles (Stavreva et al., 2009). Therapeutically, patients are typically treated with glucocorticoid replacement therapy administered in two or three divided doses each day, in an attempt to mimic circadian cortisol rhythm. One limitation of this is that the morning peaks occur later than they wake up, such that their circadian curve shifts with morning peaks occurring after they wake (Crown and

Lightman, 2005, Scott et al., 1978). Further, bolus delivery of glucocorticoid replacement therapy produces smooth hormone levels rather than mimicking the underlying pulsatile ultradian rhythm. This is thought to result in relatively constant glucocorticoid receptor occupancy, and abnormal glucocorticoid-dependent gene transcription. It has been proposed that this may contribute to side effects associated with the use of glucocorticoids, and may even be a causal factor for the excessive morbidity and mortality of patients on glucocorticoid regimes. Near physiological patterns of both circadian and ultradian rhythms can be reproduced in humans using a specialized pump and simple subcutaneous infusion technique (Russell et al., 2014).

Posttranscriptional regulation of mRNA levels of glucocorticoid responsive genes such as the ‘period circadian protein homolog’ (*Per1*) gene, may be important for regulating the circadian rhythm of glucocorticoids (Doherty and Kay, 2012, Koike et al., 2012, Morf et al., 2012). *Per1* is rhythmically expressed in the SCN. In a study of adrenalectomised rats treated with corticosterone pulses, each glucocorticoid pulse was associated with transient GR activation in neurons in the hippocampus, and a ‘pulse’ of *Per1* transcription. This suggests that glucocorticoids can regulate *Per1* in areas of the brain with high expression of GR (Conway-Campbell et al., 2010).

Glucocorticoid pulse characteristics (eg. amplitude, frequency and pattern) can be highly variable, both within and between individuals, and changes in the pattern of ultradian pulsatility are associated with transitions throughout the lifespan, physiological or pathological states. Disruptions in cortisol pulse characteristics have been described in pathological conditions such as psychotic and depressive states (Young et al., 1994, Young et al., 2004, Deuschle et al., 1997), where elevated troughs particularly at the nadir result in a flattened circadian rhythmicity (Young et al., 1994). In Cushing’s syndrome, the normal variation of cortisol is diminished due to elevations in cortisol levels during the quiescent period (van Aken et al., 2005, Boyar et al., 1979). In other disorders

that are less obviously related to glucocorticoids such as Parkinson's, Huntington's, obstructive sleep apnoea and panic disorders, the HPA axis shows changes in pulse characteristics (Henley et al., 2009b, Hartmann et al., 1997, Abelson and Curtis, 1996, Aziz et al., 2009). Disruption in cortisol pulsatile secretion has been observed during the post-surgical period following cardiac surgery (Gibbison et al., 2015).

Currently it is not known whether the disorganisation in pulsatile pattern is the cause or the consequence of the various diseases. In general, daily variations in glucocorticoid hormone concentrations are thought to be fundamental for the maintenance of physiology, and overall metabolic, cognitive and behavioural well being (Young et al., 2004, Herbert et al., 2006, de Kloet et al., 2005, Dallman et al., 2003).

Dysregulation of ultradian rhythmicity is also apparent in stress-related disease (Deuschle et al., 1997, Hartmann et al., 1997), although its precise function still remains unknown (Young et al., 2004, de Kloet and Sarabdjitsingh, 2008, Lightman et al., 2008). Studies using adrenalectomised animals replaced with various regimes of corticosterone have demonstrated that there are interactions between the HPA axis response to stress and the circadian rhythm (Akana et al., 1992, Dallman et al., 2004, Jacobson et al., 1988, Akana et al., 1988). There is also now evidence that stress responsiveness is related to ultradian hormone profiles. Analysis of hormone profiles of rats exposed to noise stress have demonstrated that the animals only responded with an increase in corticosterone when the stressor occurred during the ascending phase of an ultradian pulse (Windle et al., 1998b). Animal studies also suggest that it is the pulsatile pattern as opposed to absolute concentrations of corticosterone that determine subsequent responsiveness to stress (Sarabdjitsingh et al., 2010a, Sarabdjitsingh et al., 2010b), and that after a disruption to the ultradian rhythm, that return of normal behaviour may lag behind the return of normal HPA axis (Sarabdjitsingh et al., 2010b). Taken together, these studies suggest that it is the pulsatile pattern

rather than the absolute concentrations of corticosterone exposure that determines subsequent responsiveness to stress, and this has obvious implications for understanding the pathogenesis of stress-related disease.

Given the relationship between glucocorticoid pulsatility, transcriptional regulation and the possible implications for disease processes, a better understanding of this ultradian rhythm may assist both diagnosis and treatment of conditions associated with altered cortisol levels, whilst also aiding the development of novel therapeutic interventions. Current therapies which tend to be based on a bolus or slow-release of therapeutic glucocorticoid may lead to a non-pulsatile level of circulating cortisol, that may cause dysregulation of glucocorticoid responsive genes.

1.2.1.3 Cortisol Sampling Methods and Indices

Cortisol is a dynamic hormone, and levels fluctuate throughout the day, due to the combination of the diurnal rhythm, the ultradian rhythm, and the response to physiological and psychological stressors. Light exposure and food consumption have also been linked to cortisol secretion (Rosmond et al., 2000, Scheer and Buijs, 1999).

Daily circulating cortisol concentrations can be measured by sampling saliva, blood (plasma or serum), or urine. Saliva cortisol indexed 'free' or unbound cortisol, while blood and urine measurements reflect the total cortisol. A longer-term measure of cortisol concentrations can be assessed from hair, which stores cortisol, and has been shown to correlate with 24-hour glucocorticoid metabolites of urinary free cortisol (UFC) (Sauve et al., 2007). These methods of sampling can be used to assess different measures of cortisol activity. These include the cortisol awakening response (CAR), diurnal slope, total daily output, cortisol reactivity, and longer-term output.

The CAR (**Figure 1-3**) is the rise in cortisol levels that occurs early in the morning at the time of waking (Pruessner et al., 2003). Abnormalities of this index could include a ‘blunted’ or ‘exaggerated’ response, and such irregularities have been linked with mental and physical health ailments (Bjorntorp and Rosmond, 2000, Fries et al., 2009).

A greater CAR has also been demonstrated in individuals who are chronically stressed (Schulz P, 1998). The CAR is normally followed by a declining diurnal slope (Pruessner et al., 2003) (**Figure 1-3**). The significance of a flatter diurnal slope, which may have been preceded by a lesser CAR, or have failed to decline sufficiently by the evening, is that such patterns in cortisol change during day-time have been linked to negative physical and mental health outcomes (Fries et al., 2009). To assess the diurnal slope, multiple cortisol measurements obtained at various time-points throughout the day are required. As this can be burdensome for patients, some studies use either a single time point, or examine portions of the slope such as the ‘early decline’ or ‘late decline’.

Total daily cortisol output is typically a cumulative measure of cortisol concentrations throughout a day. This can be measured from serum, plasma, 24-hour collection of urine, or multiple days of diurnal blood or saliva sampling. Multiple sampling for a diurnal measure can be used to generate a profile of cortisol concentrations, from which the area under the curve (AUC) can be calculated.

‘Cortisol reactivity’ describes the cortisol concentrations in response to acute physical or psychosocial stress.

Longer-term cortisol output can be assessed through measurements of hair cortisol. Hair stores cortisol and grows at approximately one centimetre per month, thus can give an indication of cortisol concentrations over a longer period of time (Stalder and Kirschbaum, 2012).

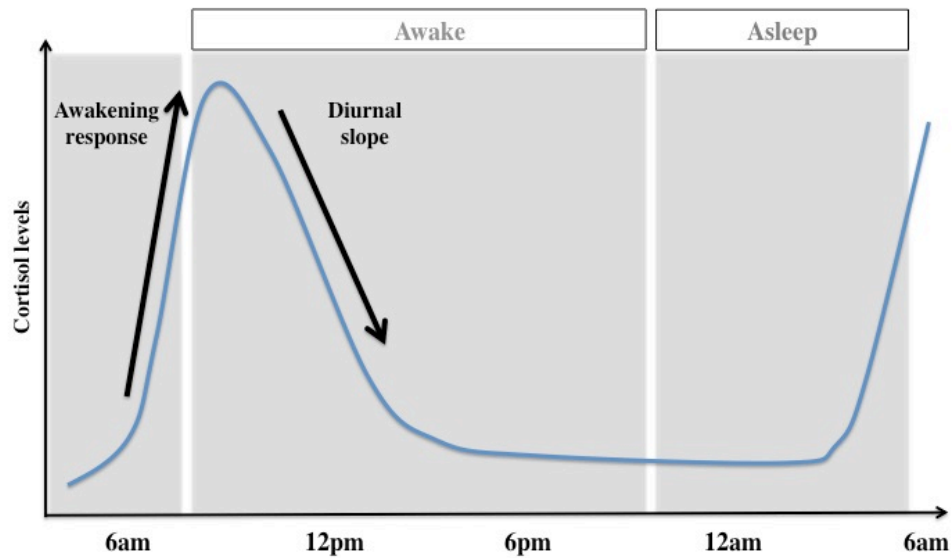


Figure 1-3. Twenty-four hour cortisol profile demonstrating awakening response and diurnal slope.

1.2.2 The HPA-axis in Obesity

Altered levels of HPA axis hormones have been reported in association with different weight statuses. Hypocortisolism is associated with weight loss, such as in Addison's disease (Lovas and Husebye, 2007), or after adrenalectomy (Bethune, 1989). In animal studies, adrenalectomy prevents genetic and diet-induced obesity (Okada et al., 1993). Hypercortisolism, such as in Cushing's syndrome, is associated with weight gain (Hankin et al., 1977). In adipose tissue, glucocorticoids promote the differentiation of pre-adipocytes into mature

adipocytes and also increase lipoprotein lipase activity. Together these promote adipose tissue increase and weight gain (Tataranni et al., 1996).

However, there is conflict in the literature about whether obesity promotes dysregulation of the HPA axis or vice versa. Studies examining this have measured different parameters of HPA axis activity including general cortisol activity using saliva, blood, urine and hair, and have tested adipocyte cortisol metabolism by assessing 11 β -HSD1 and 11 β -HSD2 in adipose tissue biopsies. Variation in study outcomes is compounded from the difference in anthropometric indices selected, which include generalised obesity indexed by BMI, and abdominal adiposity indexed by measures such as the waist to hip ratio (WHR), waist circumferences, and sagittal diameter.

Studies of the relationship between obesity (generalised or abdominal) and the cortisol awakening response include reports of a negative relationship and blunted CAR (Ljung et al., 1996, Champaneri et al., 2013, Ranjit et al., 2005, Ursache et al., 2012, Duclos et al., 2005), reports of a positive relationship and exaggerated CAR (Wallerius et al., 2003, Steptoe et al., 2004, Therrien et al., 2007), and reports of no relationship between obesity and CAR (Filipovsky et al., 1996, Kumari et al., 2010). A recent systematic review examining these studies suggested that when considering the strength of the methodologies, that a blunted CAR in obesity is the more reliable perturbation (Incollingo Rodriguez et al., 2015).

Obesity has been linked to a flatter decline of the diurnal slope throughout the day, using both BMI (Champaneri et al., 2013) and WHR (Steptoe et al., 2004). Evening cortisol has also been reported to be higher with increasing BMI in men (Abraham et al., 2013), but not in women (Ranjit et al., 2005). Imprecision of measuring the diurnal slope between studies, with use of single time proxies may contribute to these inconsistencies (Incollingo Rodriguez et al., 2015). One study reported a relationship between abdominal obesity, a flatter diurnal slope, and a

higher risk of cardiovascular disease, stroke and type 2 diabetes (Rosmond and Bjorntorp, 2000).

An increase in urinary glucocorticoid metabolites has been reported in association with increased BMI (Stewart et al., 1999, Rask et al., 2001, Rask et al., 2002) and with increased WHR (Marin et al., 1992), suggesting that obesity is associated with increased renal clearance of glucocorticoid metabolites. Hypocortisolism has also been reported in obesity, with significantly lower levels of urinary free cortisol in association with increased WHR (Vicennati and Pasquali, 2000). There are also studies describing no relationship between urinary free cortisol output when testing with BMI (Champaneri et al., 2013) or WHR (Duclos et al., 2005).

Greater consistency can be seen in literature describing the relationship between obesity and cortisol reactivity to stressors. This suggests that abdominally obese individuals demonstrate greater cortisol reactivity to stress than those with peripheral body fat (Marin et al., 1992, Rosmond et al., 1998, Duclos et al., 2005, Vicennati and Pasquali, 2000). One study showed that women with low BMI but high WHR had significantly higher levels of cortisol than women with low BMI and low WHR (Epel et al., 2000).

A significant positive relationship has been reported between BMI and hair cortisol (Stalder et al., 2012, Wester et al., 2014), but these studies did not measure abdominal obesity.

Studies of adipocyte cortisol metabolism in obese females have described a positive relationship between 11 β -HSD1 expression with BMI (Rask et al., 2002, Desbriere et al., 2006, Westerbacka et al., 2003, Mariniello et al., 2006, Munoz et al., 2009), waist circumference (Engeli et al., 2004, Desbriere et al., 2006), and sagittal diameter (Desbriere et al., 2006); and that 11 β -HSD2 is lower in abdominally obese females than non-abdominally obese, measured by waist

circumference (Engeli et al., 2004). Taken together, these findings suggest there is a greater potential for cortisol regeneration in the adipose tissue of obese.

Hepatic 11β -HSD1 activity may be impaired in the human liver. In obese men, greater glucocorticoid excretion as metabolites of cortisone than cortisol was reported (Rask et al., 2001), and in obese women, similar findings were reported with a higher cortisone: cortisol ratio (Rask et al., 2002) than non-obese controls. Incollingo Rodriguez et al., 2015 suggested that increased intracellular adipocyte cortisol regeneration may contribute to lower central cortisol secretion, and a lesser hepatic regeneration may contribute to a compensatory activation of the HPA axis in obesity, promoting metabolic disease and the obese phenotype (Rask et al., 2001, Rask et al., 2002).

1.3 The HPA Axis in Pregnancy

1.3.1 Gestational Changes to the HPA Axis

During normal human pregnancy, a number of endocrine changes lead to dramatic activation of the maternal HPA axis, which result in an exponential increase in maternal cortisol levels (Duthie and Reynolds, 2013, Lindsay and Nieman, 2005). The placenta has significant endocrine properties, and secretes CRH from the second trimester onwards, which contributes to maternal cortisol production and creates a complex feed-forward loop with cortisol also stimulating CRH from the placenta (Lindsay and Nieman, 2005, Jung et al., 2011). Estrogen and progesterone are also secreted by the placenta, and may mediate levels of free, bioavailable cortisol (Jung et al., 2011, MacLaughlin et al., 1972). Estrogen stimulates hepatic production of CBG during pregnancy (Mazer, 2004), which contributes to an increase in both bound and 'free' cortisol. Progesterone can act to displace cortisol from CBG (MacLaughlin et al., 1972). Although the circadian rhythm of cortisol release is maintained during pregnancy

(Entringer et al., 2010, Nolten et al., 1980, Nolten and Rueckert, 1981, Cousins et al., 1983), the cortisol awakening response and physiological response to stress become attenuated as gestation advances (Lindsay and Nieman, 2005). One study, using 30-minute serum sampling, demonstrated that cortisol pulses are maintained in the third trimester of human pregnancy (Magiakou et al., 1996), but how and whether the ultradian rhythm of cortisol secretion changes across gestation is unknown.

1.3.2 *In Utero* Cortisol Exposure

1.3.2.1 Fetal Growth

Glucocorticoids are vital for normal fetal growth and organ maturation. Prophylactic corticosteroids administered antenatally to women at risk of delivering preterm are known to accelerate lung maturation and reduce the incidence of respiratory distress syndrome (RDS) in both extremely preterm (Morgan et al., 2016) and nearer term babies (Saccone and Berghella, 2016).

In addition to promoting maturation of fetal organs, increased cortisol levels may also increase availability of glucose for the developing fetus, via mobilisation of substrates for hepatic gluconeogenesis (Reynolds et al., 2003). This would suggest that increased fetal glucocorticoid exposure may lead to increased fetal growth. However, contrary to this suggestion both human and animal studies indicate that fetal overexposure to glucocorticoids is in fact linked with fetal growth restriction (Stewart et al., 1995). Timing of fetal glucocorticoid exposure may also be critical; amniotic fluid cortisol levels (a surrogate marker for fetal glucocorticoid exposure) at 15-18 weeks are negatively correlated with birthweight ($r=-0.25$, $p<0.001$) and gestational age ($r=-0.18$, $p<0.05$) (Baibazarova et al., 2013). This suggests that cortisol exposure in the second trimester of human pregnancy may influence fetal growth.

Maternal cortisol levels have also been shown to predict offspring birthweight. High maternal serum cortisol at 20 weeks gestation has been shown to be negatively associated with offspring birthweight (Goedhart et al., 2010). Higher salivary cortisol waking response at 13-18 weeks gestation is negatively correlated with shorter offspring length at birth (Bolten et al., 2011). At 36 weeks both higher morning cortisol and a steeper morning decline in cortisol levels are associated with lower birthweight (Kivlighan et al., 2008). Taken together, these studies suggest that a flattening of the diurnal rhythm of cortisol secretion may protect the fetus from overexposure to glucocorticoids during the morning peak.

1.3.2.2 Length of Gestation

The mechanisms that determine the length of gestation and the onset of parturition multifactorial, and are not well understood, but several studies suggest that the HPA axis plays a role in these processes.

Maternal plasma CRH, which predominately originates from the placenta during pregnancy, has been linked to the timing of birth (Wolfe et al., 1987, Warren et al., 1992, Hobel et al., 1999, Wadhwa et al., 1998). Levels of CRH in the maternal circulation increase exponentially with advancing gestation, and peak at delivery. At the end of human pregnancy, production the circulating binding protein for CRH (CRHBP) declines, thus increasing the bioavailability of CRH (Linton et al., 1990, Linton et al., 1993). CRH has been shown to induce production of chemokines and cytokines *in vitro*, in myometrium at term and subsequently results in the cascade of inflammation in the uterus. The inflammation induced by CRH may lead to the onset of parturition (You et al., 2014). Preterm labour is associated with a more rapid increase in CRH levels (Torricelli et al., 2011, McLean et al., 1995), while women with prolonged or post-term pregnancies have a slower rise in CRH (Torricelli et al., 2006). Taken

together, these findings suggest that CRH plays a key role in governing the length of gestation and triggering the onset of labour.

An early abnormal rise of endogenous cortisol at 15-19 weeks of gestation was shown to be associated with preterm labour (Sandman et al., 2006) whereas the gestational increase in fetal cortisol appears to be blunted in the postmature fetus (Nwosu et al., 1975). In the 1960s exogenous glucocorticoids were shown to induce labour in sheep (Liggins, 1968), and subsequent clinical studies investigated whether glucocorticoids also induced labour in humans (Craft et al., 1976, Katz et al., 1979, Nwosu et al., 1976, Elliott and Radin, 1995). Synthetic glucocorticoids administered into amniotic fluid were more effective at initiating labour than when systemically administered (Craft et al., 1976). This suggests that glucocorticoids may have an effect in the intrauterine tissues to initiate labour. Intramuscular administration of glucocorticoids has not been shown to result in delivery in humans.

The synthetic glucocorticoids used in clinical practice to promote fetal lung maturation and in clinical trials to induce labour (dexamethasone and betamethasone), are, unlike endogenous cortisol, not substrates for placental 11 β -HSD2 (Seckl, 1997), which inactivates cortisol into cortisone (described in more detail in section 1.3.3.3). These synthetic glucocorticoids therefore pass through the placenta and into the fetal compartment intact, and contribute to a negative feedback effect on the fetal HPA axis. The result of this is a decrease in fetal cortisol synthesis, and subsequent reduction in placental estrogen synthesis (Craft et al., 1976, Ogueh et al., 1999, Ohrlander et al., 1977). This negative feedback effect may negate any effect of systemic administration of synthetic glucocorticoids on induction of labour. Endogenous circulating cortisol increases throughout gestation. Further, fetal membranes and the decidua express increasing 11 β -HSD1 (which regenerates cortisol from cortisone) with advancing gestation (Alfaidy et al., 2003). These tissues are considered to be important sources of extra-adrenal cortisol within intrauterine tissues (Murphy,

1977), and increasingly regenerate cortisol with advancing gestation (Tanswell et al., 1977).

1.3.2.3 Cardio-Metabolic Disease

Fetal glucocorticoid exposure has been linked to the development of cardio-metabolic disease later in life. Glucocorticoids increase insulin resistance and inhibit glucose-stimulated insulin secretion from pancreatic beta cells (Reynolds and Walker, 2003). Glucocorticoids also cause hypertension, vasoconstriction and dyslipidaemia, all of which increase the risk of myocardial and cerebral infarction.

Offspring of women who received antenatal corticosteroid therapy for suspected preterm labour have been reported to have higher blood pressure at age 14 years (Doyle et al., 2000), and higher insulin levels at age 30 years (Dalziel et al., 2005). Exposure to increased levels of endogenous glucocorticoids during pregnancy as a result of reduced placental 11 β -HSD2 activity (estimated from cord blood cortisol and cortisone concentrations) associates with increased blood pressure at aged 3 years (Huh et al., 2008).

Higher maternal cortisol during pregnancy is associated with higher fat mass index in female offspring, and with lower fat mass index in male offspring (Van Dijk et al., 2012).

Studies using cohorts with birthweight records have reported that low birthweight associates with increased cortisol levels and adrenal responsiveness to ACTH, and an adverse metabolic profile in adulthood (Phillips et al., 1998, Reynolds et al., 2001, Phillips et al., 2000, Reynolds et al., 2005). This supports the hypothesis that increased exposure to glucocorticoids over the lifespan may be the link to how events *in utero* may be linked to metabolic disease later in

adulthood.

1.3.3 The Protective Role of the Placenta

During pregnancy, the placenta, which is the primary barrier between the maternal and fetal circulations, acts as the renal, respiratory, gastrointestinal, endocrine and immune systems for the developing fetus. In the 1950s and 1960s, birth defects caused by thalidomide highlighted that the placenta is not a complete barrier for drug transfer. Subsequent research has focused on characterising the mechanisms by which drugs and nutrients pass across the placenta.

1.3.3.1 Placental Structure and Development

Placental development begins with decidualisation of the uterus, which means that the uterus is ready for implantation of the embryo. During decidualisation uterine stromal cells are converted into large, secretory decidual cells, and macrophages and lymphocytes are recruited (Malassine et al., 2003). In humans the first signs of decidualisation can occur before conception, and as early as day 23 of the menstrual cycle. At this time, spiral arteries become prominent in the endometrium (Kliman, 2000). After the blastocyst adheres to the decidua, the blastocyst differentiates into an embryoblast (which later develops into the embryo and fetus), and the outer layer of the blastocyst is the trophoblast, which later becomes the placenta.

The trophoblast further divides into two layers: the underlying ‘cytotrophoblast’, and the overlying ‘syncytiotrophoblast’ layers, which is a multinucleated cell layer covering the surface of the placenta and therefore has a role in the ‘barrier’ role of the placenta. As gestation advances the placenta continues to grow as a

discoid-shaped organ. By the end of the first trimester of pregnancy, blood supply from the maternal circulation is complete.

In human pregnancy, the chorionic villus (the basic structural and functional unit of the mature placenta) is established as early as day 21 after ovulation (Malassine et al., 2003). These are an inner network of vascular projections of fetal tissue surrounded by the outer syncytiotrophoblast (directly in contact with the intervillous space containing maternal blood), and the inner cytotrophoblast. The intervillous space contains maternal blood. With advancing gestation as the villi mature, there is a reduction in the cytotrophoblast and by term, there is only a single layer of syncytiotrophoblast separating maternal blood from the fetal capillaries. Spiral arteries from the uterus penetrate the placenta and supply the intervillous space with maternal blood.

There are two umbilical arteries that arise from the fetal internal iliac arteries that carry deoxygenated blood from the fetus via the umbilical cord to the placenta. These umbilical arteries divide into chorionic arteries and end as capillaries within the chorionic villi. Substances contained within the maternal blood pass in the intervillous space pass through the syncytiotrophoblast and fetal capillary endothelium into the fetal blood. Fetal capillaries drain into chorionic veins that then empty into a single umbilical vein.

The placenta grows with advancing gestation and at term is about 15-20 cm in diameter, 2-3 cm thick and has a surface area of up to 15 m² (Moore KL, 2008).

1.3.3.2 Placental Function

The placenta is the primary interface between the maternal and fetal bloodstream. The syncytiotrophoblast performs multiple functions; it's primary

role is in absorption, exchange of gases, nutrients and waste, and hormone production.

Infant size at birth is thought to be determined by fetal nutrient supply (Fowden et al., 2006), which is influenced by placental size, morphology and capacity for nutrient transport (Fowden and Forhead, 2004). Placental weight is positively correlated with fetal weight near term in a number of species (Baur, 1977, Fowden et al., 2009). Placental production of nutrients and hormones are also known to affect fetal growth (Fowden and Forhead, 2004). A growing body of evidence suggests that specific alterations in placental transporter function are implicated in fetal growth pathologies (Jansson and Powell, 2006, Sibley et al., 2005).

Alterations in the HPA axis during pregnancy may have an impact on placental nutrient transport. Glucocorticoids have been shown to down regulate placental glucose transporters (GLUT1 and GLUT3) (Hahn et al., 1999), to reduce activity of human placental lipoprotein lipase, which is involved in lipid transport to the fetus (Magnusson-Olsson et al., 2006) and to up regulate System A Amino Acid Transporter 2 (SNAT2). CRH has been shown to down regulate system A activity (Giovannelli et al., 2011) and down regulate placental GLUT3 (Gao et al., 2012). Estradiol, estriol and progesterone have also been shown to inhibit placental monosaccharide transport (Johnson and Smith, 1980). Taken together these findings suggest that alterations in the HPA axis during pregnancy may influence availability of nutrients for the developing fetus and may be an underlying mechanism for glucocorticoid associated growth abnormalities.

As previously mentioned, the placenta has significant endocrine properties and produces a number of steroid and peptide hormones including human chorionic lactogen, human placental lactogen, human growth hormone variant, estrogen, progesterone and CRH.

1.3.3.3 Placental 11 β -HSD2

The placenta is equipped with several 'defence mechanisms' which are thought to help prevent the fetus from the harmful effects of overexposure to glucocorticoids, such as fetal growth restriction (Stewart et al., 1995). Glucocorticoids are thought to be lipophilic, meaning that they can freely cross the placenta between the maternal and fetal compartments. Maternal plasma cortisol is significant as an independent predictor of amniotic fluid cortisol, after adjusting for maternal age, gestational age and time of collection (Sarkar et al., 2008), and increases with increasing gestation (Sarkar et al., 2008, Baibazarova et al., 2013, Gitau et al., 1998, Glover et al., 2009).

One relatively well characterised 'defence mechanism' is the cortisol inactivating enzyme, 11 β -HSD2, which converts active cortisol into inactive cortisone, thus limiting fetal glucocorticoid exposure. 11 β -HSD2 is present at higher concentrations in the placenta with advancing gestation, and its activity falls dramatically at term, to correspond with fetal lung maturation and parturition (McTernan et al., 2001, Murphy and Clifton, 2003, Benediktsson et al., 1993). Due to the action of 11 β -HSD2, fetal glucocorticoid levels are up to 10-fold lower than maternal levels (Edwards et al., 1993), although it is thought that up to 20% of maternal glucocorticoids cross to the fetal compartment (Benediktsson et al., 1997).

Both human and animal studies have suggested that the efficiency of placental 11 β -HSD2 may be reduced by specific dietary substances, or the presence of inflammation, hypoxia and stress. Reduced enzyme efficiency would allow a greater transplacental passage of cortisol to the developing fetus (Seckl, 2004, Cottrell and Seckl, 2009). Given that maternal cortisol levels are considerably higher than fetal levels, even subtle changes in placental 11 β -HSD2 activity would have the potential to significantly alter fetal glucocorticoid exposure (Gitau et al., 1998, Gitau et al., 2001).

In animal studies, both pharmacological and genetic modifications to reduce 11 β -HSD2 activity have been shown to associate with lower birthweight (Seckl, 2004, Cottrell and Seckl, 2009). Similarly lower levels of 11 β -HSD2 in human placenta associates with lower birthweight (Stewart et al., 1995, McTernan et al., 2001), and babies homozygous for deleterious mutations of 11 β -HSD2 weigh on average 1.2 kg less than heterozygote siblings (Dave-Sharma et al., 1998). Women who consume large quantities of liquorice during pregnancy (which contains glycyrrhizin, an 11 β -HSD inhibitor) have shorter gestation (Strandberg et al., 2001, Strandberg et al., 2002). Taken together, these studies suggest that placental 11 β -HSD2 activity, which regulates fetal glucocorticoid exposure, is critical for fetal growth and length of gestation.

Placental biology may also be altered by exposure to stress *in utero*. In late pregnancy, maternal anxiety associates with up regulation of GR and MR mRNA (Reynolds et al., 2015) and down regulation of 11 β -HSD2 (O'Donnell et al., 2012). Together, these may increase placental glucocorticoid sensitivity and increase fetal cortisol exposure.

1.4 Aims, Hypothesis and Outline of Thesis

With recognition that the prevalence of obesity and maternal obesity is increasing, and an increasing body of evidence describing both short-term pregnancy outcomes, and longer-term offspring health outcomes; studies investigating the underlying mechanisms in obese related pregnancy outcomes are an important focus for current research.

Glucocorticoids are implicated in both fetal growth and length of gestation. Further, epidemiological, clinical and laboratory studies support the hypothesis that fetal exposure to glucocorticoids *in utero* is a mediator of longer-term programming of cardiovascular, metabolic and neuroendocrine disorders in adult

life (Seckl, 2004, Reynolds, 2013) (**Figure 1-4**). Although inconclusive, available evidence suggests that non-pregnant obesity is associated with dysregulation of the HPA axis, and there is limited evidence from two recent studies that reported lower cortisol in obese compared with lean pregnant women, suggesting that HPA axis dysregulation may be maintained in obese during pregnancy (Putignano et al., 2001, Goedhart et al., 2010, Berglund et al., 2016). In 2010, Goedhart et al., reported findings from the Amsterdam Born Children and their Development cohort (Goedhart et al., 2010). This large prospective cohort study (n=2810 pregnant women, of which 105 women had a BMI ≥ 30 kg/m²) examined the relationship between maternal cortisol with birthweight and risk of offspring being small for gestational age. A single measurement of maternal total serum cortisol was obtained at 13 weeks' gestation, demonstrated that maternal cortisol was significantly lower with increasing maternal BMI (p<0.001). More recently in 2016, Berglund et al., reported findings from an observational cohort study of 331 pregnant women (normal weight n=132, overweight n=56 and obese n=64). Maternal serum cortisol was significantly lower in obese than normal weight women at 24 weeks' (p=0.05) and at delivery (p=0.007).

Further studies are required to corroborate these findings, to investigate the effects of maternal obesity on other indices of cortisol activity, to test associations with short- and longer-term clinical outcomes, and to identify underlying mechanisms.

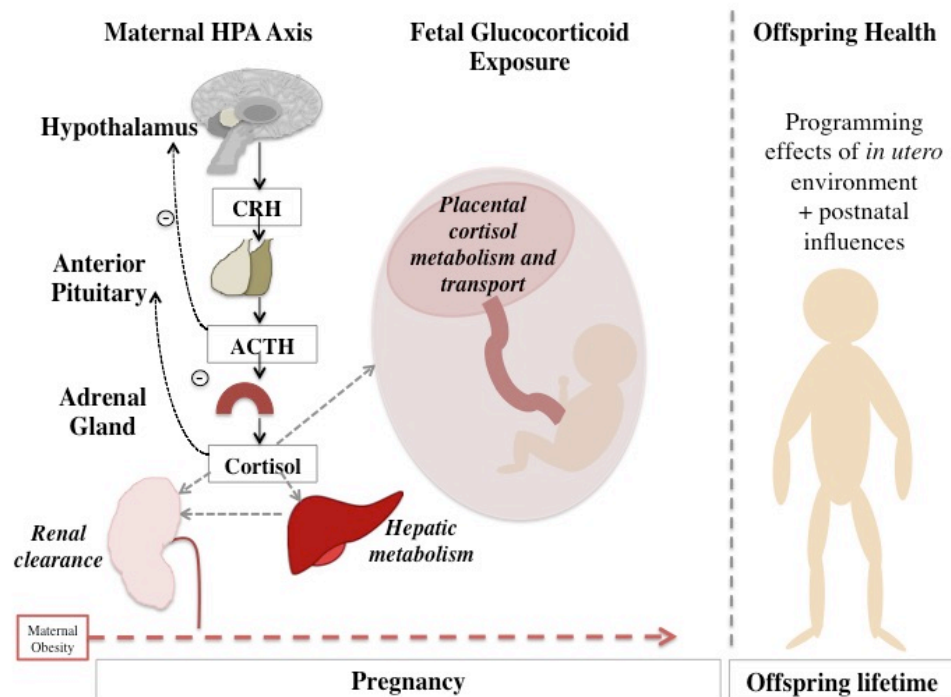


Figure 1-4. Possible *in utero* programming effect via influence of maternal obesity on maternal HPA axis, thus affecting fetal glucocorticoid exposure.

This Thesis aimed to characterise HPA axis activity in obese pregnancy, and to identify possible underlying mechanisms in its regulation. The overarching hypothesis was that HPA axis activity is dysregulated in maternal obesity, and that this may be associated with altered release, clearance and placental metabolism of cortisol, and with clinical outcomes related to fetal size and length of gestation. This hypothesis was tested in prospective longitudinal pregnancy cohorts of women with severe obesity and lean controls (**Chapters 3 and 5**), and using pregnancy blood samples obtained at the time of delivery (**Chapters 4 and 6**). This Thesis contains an overall methods chapter (**Chapter 2**) and four results chapters (**Chapters 3 – 6**):

In **Chapter 3**, the effects of maternal obesity on the maternal HPA axis are explored through measurement of circulating cortisol, CBG, CRH, estrogens (estradiol (E2) and estriol (E3)), and progesterone (P4) throughout pregnancy in a cohort of very severely obese and lean women. Associations with clinical outcomes related to birthweight and length of gestation were tested. It was hypothesised that HPA axis hormones would be lower in obese throughout pregnancy, and would associate with increased fetal size and longer gestation.

In **Chapter 4**, the effects of maternal BMI on maternal and fetal glucocorticoids at delivery are assessed by measuring cortisol, cortisone, corticosterone and 11-dehydrocorticosterone from samples of maternal and cord blood obtained at delivery. It was hypothesised that maternal and cord glucocorticoids would be lower at delivery.

In **Chapter 5**, the effects of both maternal BMI and pregnancy gestation on the ultradian rhythm of glucocorticoids are investigated through a longitudinal study of obese and lean pregnant, and non-pregnant women. It was hypothesised that ultradian pulse characteristics would be altered in obese compared with lean subjects, and would be altered with advancing gestation of pregnancy.

In **Chapter 6**, placental cortisol transport is studied using a dual perfused *ex vivo* placental perfusion model combined with mathematical modeling. A model of placental cortisol transfer and metabolism was developed which can be used in future studies to determine the impact of changes in the maternal-fetal environment.

Chapter 2

Materials and Methods

2.1 Clinical Methods

2.1.1 Tommy's Centre for Maternal and Fetal Health Research

The work in this Thesis is based on clinical research funded by Tommy's the Baby Charity, and has been carried out at the Tommy's Centre for Maternal and Fetal Health Research at the University of Edinburgh. The Edinburgh Tommy's Centre work includes studying the impact of maternal obesity on fetal exposure to glucocorticoids *in utero*.

2.1.2 Recruitment Methods

Participants were recruited from the NHS Lothian Health board region.

Obese pregnant women were recruited from the Metabolic Antenatal Clinic (MAC, **Chapters 3 and 5**); this is a pan-Lothian clinic held at the Royal Infirmary of Edinburgh, that provides specialized and Consultant-led multidisciplinary care for severely obese pregnant women with a BMI > 40 kg/m² at booking. The MAC was launched at the Simpson's Centre for Reproductive Health in 2008, initially funded by Tommy's, and now funded by NHS Lothian. Normal weight (booking BMI 18.5 – 25 kg/m²) pregnant 'controls' were recruited from community Antenatal Clinics in Edinburgh (**Chapters 3 and 5**).

Non-pregnant obese (BMI > 30 kg/m²) participants were recruited from Bariatric Surgery Clinics in NHS Lothian (**Chapter 3**) and community weight

management clinics (**Chapter 5**). Non-pregnant, normal weight (BMI 18.5 – 25 kg/m²) volunteers were recruited as volunteers responding to advertisements (**Chapters 3 and 5**).

2.1.3 Edinburgh Clinical Research Facility

The Edinburgh Clinical Research Facility is a purpose-built unit for clinical research at the Royal Infirmary of Edinburgh. It is a joint venture between the University of Edinburgh and NHS Lothian, staffed by experienced research and nursing staff. This facility was used for participant study visits in **Chapters 3 and 5**.

2.1.4 Edinburgh Reproductive Tissue BioBank

Biological samples from pregnant women (**Chapters 4 and 6**) were obtained via the Edinburgh Reproductive Tissue BioBank (ERTBB; ethical approval REC09/S0704/3). The ERTBB stores anonymised tissue specifically collected for pregnancy research from women who give written consent, by trained research midwives and research technicians. The majority of samples collected at the time of elective Caesarean section. Tissue samples are linked to a database containing clinical records.

2.2 Clinical Studies

The work presented in this Thesis relates to the following studies and approvals:

1. Hormones in Pregnancy (HIP; ethical approval 08/S1101/39).
Chief Investigator, Professor Rebecca Reynolds.

2. Altered Metabolic Processes in Obese Pregnant Women (AMPOP; ethical approval 09/S1103/6). Chief Investigator Professor Jane Norman; Principle Investigator, Dr Sarah Barr.
3. The Effect of Maternal Weight and Gestation on Cortisol Pulsatility in Pregnancy (PIPa; ethical approval 13/SS/0176). Chief Investigator Professor Rebecca Reynolds; Principle Investigator Dr Laura Stirrat.

2.2.1 Hormones in Pregnancy HIP

Obese pregnant women ($\text{BMI} \geq 40 \text{ kg/m}^2$) were recruited from the metabolic antenatal clinic, and normal weight ($\text{BMI} 18.5 - 25 \text{ kg/m}^2$) from the Lauriston community antenatal clinic, to participate in a longitudinal study of obesity in pregnancy from 2008 – 2013. **Figure 2-1** illustrates the protocol of this prospective longitudinal study. Trained research nurses and midwives carried out recruitment and conducted anthropometric measurements. Participants completed a demographic information questionnaire that covered demographics, social history and medical history prior to pregnancy. Fasting venous blood samples were obtained at 16, 28 and 36 weeks of gestation, and at 3-6 months postnatal. Three saliva samples were obtained at 16 weeks gestation in a subset of women at bedtime, at waking and at 30 minutes after waking, to assess the cortisol awakening response. Eligible women were aged 18-45 years and were Caucasian. Exclusion criteria were diabetes, active endocrine disorders, use of glucocorticoid medication and multiple pregnancy. Clinical outcomes were obtained from medical records.

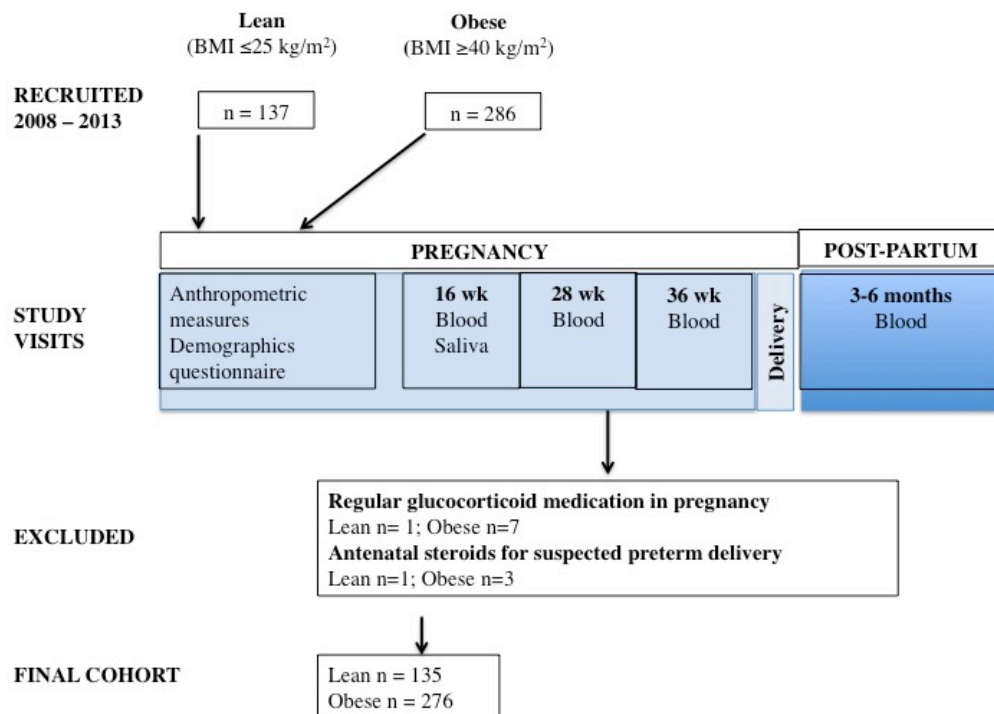


Figure 2-1. Flow Chart of Hormones in Pregnancy (HIP) Study

This longitudinal study of obesity in pregnancy forms the basis of **Chapter 3** in this Thesis.

Key: wk (weeks gestation)

2.2.2 Altered Metabolic Processes in Obese Pregnant Women AMPOP

Obese pregnant women (BMI ≥ 40 kg/m²) were recruited from the MAC, and normal weight (BMI 18.5 – 25 kg/m²) from the Lauriston community antenatal clinic, from 2009 – 2012. Non-pregnant volunteers were recruited following response to advertisement, were women who had previously attended the MAC

and had given their permission to be contacted about future studies, and from the Bariatric Surgery clinic in NHS Lothian. Recruitment was carried out by Dr Sarah Barr (Clinical Research Fellow).

Study participants provided 24-hour urine collections for analysis of urinary glucocorticoid metabolites. Samples were collected at 19 weeks and 36 weeks of gestation, and non-pregnant participants provided one collection. Eligible women were Caucasian. Exclusion criteria were diabetes, active endocrine disorders, use of glucocorticoid medication and multiple pregnancy.

2.2.3 The Effect of Maternal Weight and Gestation on Cortisol Pulsatility in Pregnancy PIPa

Obese pregnant women ($\text{BMI} \geq 40 \text{ kg/m}^2$) were recruited from the MAC, and normal weight ($\text{BMI} 18.5 - 25 \text{ kg/m}^2$) from the Lauriston community antenatal clinic, from 2014 – 2016. **Figure 2-2** illustrates the protocol of this prospective case-control study. Non-pregnant volunteers were recruited following response to advertisement, or from NHS Lothian community weight management clinics. Inclusion criteria were singleton pregnancy, normal booking ultrasound scan and Caucasian ethnicity. Exclusion criteria were smoking, diabetes at recruitment, regular glucocorticoid medication, severe mental health disorder or anaemia. Pregnant women attended for two study visits (visit 1 at 16 – 24 weeks gestation; visit 2 at 30 – 36 weeks gestation) and non-pregnant volunteers attended for one study visit in the luteal phase of the menstrual cycle. Study visits took place at the Edinburgh Clinical Research Facility.

Participants attended study visits following an overnight fast. Fasting blood samples were obtained at 10-minute intervals between 08.00h – 11.00h and between 16.00h – 19.00h via a peripheral cannula. Interstitial fluid from the subcutaneous area of the abdomen was obtained at 20-minute intervals starting

between 0830h and 0900h for 24-hours, via a portable microdialysis and automated collection device (described in greater detail: Section 2.3.4). Participants continued to wear this at home overnight, and it was collected from them the following morning.

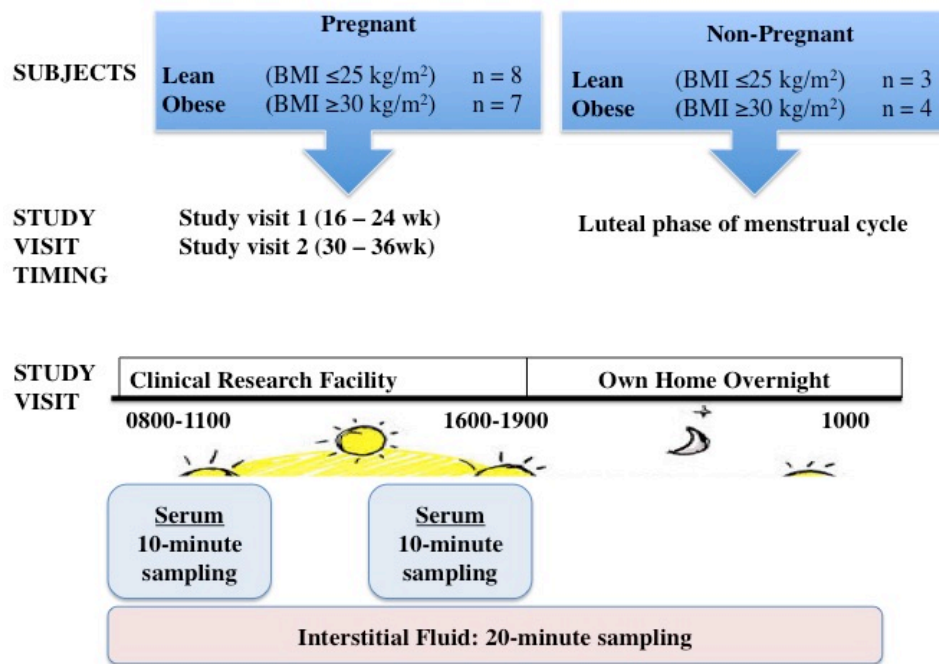


Figure 2-2. The Effect of Maternal Weight and Gestation on Cortisol Pulsatility in Pregnancy (PIP_a) Study

This case-control study forms the basis of **Chapter 4** in this Thesis. Details of the analyses are given in **Chapter 4**.

2.2.4 Studies of Blood and Tissue at Delivery

Samples of paired maternal and cord blood (**Chapter 4**), and whole placentas (**Chapter 6**) were obtained from pregnant women who had consented for storage and use of tissue in the Edinburgh Reproductive Tissue BioBank for pregnancy research. Specific inclusion and exclusion criteria are details in **Chapters 4 and 6**. Collection procedures and experimental protocols are described in sections 2.3.1 (blood) and 2.4.5 (placenta).

2.3 Tissue Collection

2.3.1 Blood

Peripheral venous blood was collected into the appropriate container (Sarstedt Monovette®, UK), for plasma (anti-coagulated with sodium EDTA), or serum (container with separation polyacrylic ester gel). Cord blood (arterial or venous) was collected immediately after delivery and cord clamping. Samples were transported on ice, and plasma or serum were separated within 30 minutes after collection by centrifugation at 1200 rpm for 10 minutes at 4 °C. Blood fractions were stored at -80 °C until analysis. Both serum and plasma are widely used for measurement of circulating cortisol levels in humans. Consistent use of either serum or plasma within a given study will reduce the potential for assay variation. The studies in **Chapters 3 and 5** collected serum samples for cortisol analysis. In **Chapter 4** plasma samples were used, as there was greater availability of plasma than serum samples, thus a larger sample size could be generated using plasma samples.

2.3.2 Saliva

Saliva samples were collected using a Salivette® collector (Sarstedt, Numbrecht, Germany); at bedtime, at waking, and 30 minutes after waking, to assess the cortisol awakening response. Samples were centrifuged and stored at -20 °C until later analysis.

2.3.3 Twenty-Four Hour Urine Collection

Urine samples were collected by Dr Sarah M Barr as part of the AMPOP study. Participants provided urine samples in a Twenty-Four Hour Urine Container 2.5 L (Medline, Oxon, UK). Total urine volume was recorded and stored in 20 mL aliquots at -80 °C until analysis.

2.3.4 Interstitial Fluid

Interstitial fluid cortisol concentrations (which are mostly free cortisol) (Sandeep et al., 2005) can be measured using microdialysis techniques. Microdialysis is an *in vivo* sampling technique for measuring endogenous and exogenous solutes in the extracellular space of tissue. A small probe equipped with a semi-permeable hollow fibre can be inserted superficially into the dermis, and perfused with a solution that forms an equilibrium, with diffusible molecules in the immediate surroundings (Schnetz and Fartasch, 2001) (**Figure 2-3**). A novel miniaturized sampling system that combines the technique of microdialysis with the ability to collect multiple samples automatically over 24 hours was developed by Professor Stafford Lightman's group (Bristol) (Bhake et al., 2013). Samples are obtained at 20-minute intervals for 24 hours, then decanted manually into marked tubes and stored at -80 °C for later laboratory analysis.

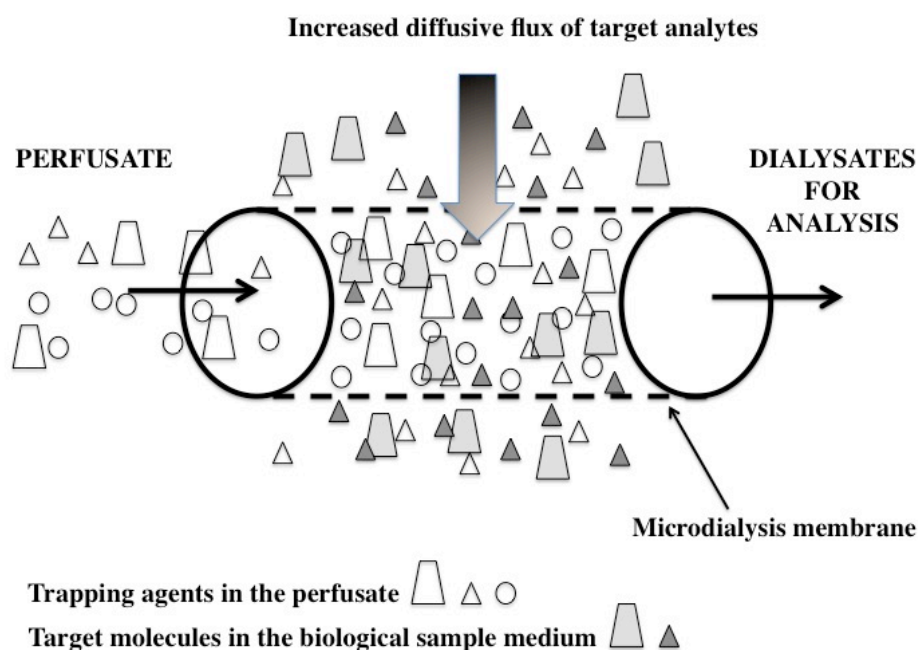


Figure 2-3. Schematic Representation of Microdialysis Membrane

2.3.5 Term Placentas

Whole placentas were obtained at delivery for placental perfusion studies, from term pregnancies (**Chapter 6**) from women undergoing elective Caesarean section at the Royal Infirmary of Edinburgh. Placentas were transported to the laboratory on ice for immediate assessment of suitability for experimental use.

2.4 Laboratory Studies

Laboratory studies and analytical techniques were performed at the Tommy's

Centre for Maternal and Fetal Health Research, or the Mass Spectrometry Core of the Edinburgh Clinical Research Facility, unless stated otherwise.

2.4.1 *Ex Vivo* Placental Perfusion

Functional studies of the human placenta are challenging, due to the potential risk of harm to a pregnant women or the developing fetus. Ethical constraints mean that *in vivo* studies of human pregnancy are restricted, so for this reason *ex vivo* experiments have a key role in human placental research. The *ex vivo* dual perfused human placental cotyledon is the only experimental model that facilitates study of placental transfer in organized human placental tissue. Briefly, this technique involves establishing a maternal and fetal circulation, and creating an environment to maintain tissue viability by supplying it with nutrients, gasses and maintaining temperature, all of which are intended to mimic physiological conditions. Perfusion of a single human placental cotyledon where the maternal and fetal circulations are separate was originally described in 1962 (Panigel, 1962), was developed further by the groups of Schneider (Schneider et al., 1972) and Miller (Miller et al., 1989), and is widely used today. The success rate of perfusion experiments in placentas that are suitable for being connected to a perfusion circuit, is only about 50% (Myllynen and Vahakangas, 2013). The perfusion method for the work presented in this Thesis was based on previous studies (Brownbill et al., 2000, Cleal et al., 2007).

Whole placentas were obtained with informed consent following elective Caesarean section of uncomplicated term pregnancies, via the Edinburgh Reproductive Tissue BioBank. Immediately following delivery of the placenta with gentle controlled cord traction, the umbilical cord was clamped and placed into a plastic bag before being transported to the laboratory on ice.

In the laboratory, placentas were examined for evidence of tissue damage during delivery. Vein and artery pairs with minimal branching that supplied a well-defined and macroscopically intact cotyledon were identified. A non-recirculating circulation was established. Fetal vessels were cannulated (fetal artery 19 G blunt needle with internal diameter 0.7 mm, and fetal vein 14 G blunt needle with internal diameter 1.6 mm; OctoInkJet, UK), sutured in place (4-0 Polysorb, Coviden, UK), and fetal circulation started with perfusion of perfusion buffer at 6 mL/min. If required, collateral vessels adjacent to the cotyledon were tied off. The perfused cotyledon was then placed with the 'fetal side' facing downwards in the perfusion chamber (custom made for a previous study and gifted for this work by Dr Fiona Denison, Reader and Honorary Consultant in Maternal Fetal Medicine, University of Edinburgh). The fetal side was covered with Parafilm® (Bemis NA, Neenah, WI, USA), to prevent any sagging or undue stretch on the vessel and placental tissue. Placental edges were trimmed, allowing a generous margin to minimize fluid leaks.

The perfused cotyledon was identified on the 'maternal side' by slight blanching. Maternal perfusion was established by introducing three blunt cannula (Cole-Parmer Instrument Company, London, UK) into the intervillous space, by penetrating the basal plate to a depth of approximately 1-2 cm. The maternal infusion was started at 14 mL/min. Maternal perfusate that returned from the intervillous space continuously drained by a venous catheter placed at the lowest level of the maternal surface, to avoid significant pooling of perfusate.

A waterbath was utilized to maintain the perfusate reservoirs at 37 °C and the tubing passed through a second water bath (also at 37 °C) before entering the perfusion chamber itself was placed inside an incubator (Hybaid, UK), also at 37 °C.

The maternal and fetal circulations were driven by a peristaltic pump (Watson-Marlow, Cornwall, UK). The different flow rates (maternal 14 mL/minute, fetal

6 mL/minute) were achieved by using different bore tubing segments for the pump (maternal bore 3 mm and fetal bore 2 mm, Elkway Precision Laboratory Consumables, Hampshire, UK). Tubing for the rest of the circuit was 2.0 mm bore (Portex®, Smiths Medical, Kent, UK). *In vitro* flow rates on the maternal and fetal sides have been estimated as being around 20 mL/min per 30 g of lobule (Kirkinen et al., 1983). However, this has been shown to result in villous oedema in *ex vivo* dual perfused models (Kaufmann, 1985).

The initial perfusate was Earle's bicarbonate buffer (EBB) with additional BSA 2 g/L added. The perfusion system was gassed using gas exchange devices (HiQ, BOC, UK). The maternal circulation was equilibrated with 95% air and 5% CO₂ (BOC, UK), and the fetal perfusate was equilibrated with 95% N₂ and 5% CO₂ (BOC, UK).

Although this model has been used experimentally for decades, there are no standardized criteria for monitoring and validation. The parameters selected to monitor the system and estimate viability in the perfusions presented in this Thesis are as follows:

Volume conservation: Fetal output was measured by collecting the fetal effluent over 5 minute intervals, knowing that the input was 6 mL/minute. Venous return flow of > 95% of the input flow was enforced throughout each perfusion (Cleal et al., 2007).

Pressure: Fetal perfusion pressure of > 70 mmHg has been shown to lead to damage of the fetal villi during an *ex vivo* perfusion (Jauniaux et al., 1991). A pressure transducer (Memscap®, Skoppum, Norway) was inserted into the fetal artery section of the circuit and continuously detected pressure that was reported by a computer program (Labchart v7.0, ADInstruments, Oxford, UK). Fetal artery pressures up to 70 mmHg were considered successful, and the perfusion was considered unsuccessful if pressure in the fetal circuit exceeded 70 mmHg.

Inability to achieve adequate circuit perfusion rates within fetal inflow pressure ranging 40 – 70 mmHg perfusion was stopped.

As the placenta suffers an ischaemic ‘insult’ injury at delivery, experiments tend to allow a 30-60 minute recovery period after the perfusion experiment has commenced to allow metabolic functions to recover. For the experiments in this Thesis, 30 minutes was considered appropriate to wash out the endogenous glucocorticoids that remained in the placenta (Benediktsson et al., 1997). Most published studies have reported perfusing placentas for 2-6 hours, but perfusions up to 48 hours have been reported (Miller et al., 1989, Heikkila et al., 2002, Woo et al., 2012). The infusion protocol for the experiments presented in this Thesis is outlined in **Figure 2-4**. On completion of the perfusion experiment, the wet weight of the perfused cotyledon was obtained and samples of perfused tissue were stored at -80 °C.

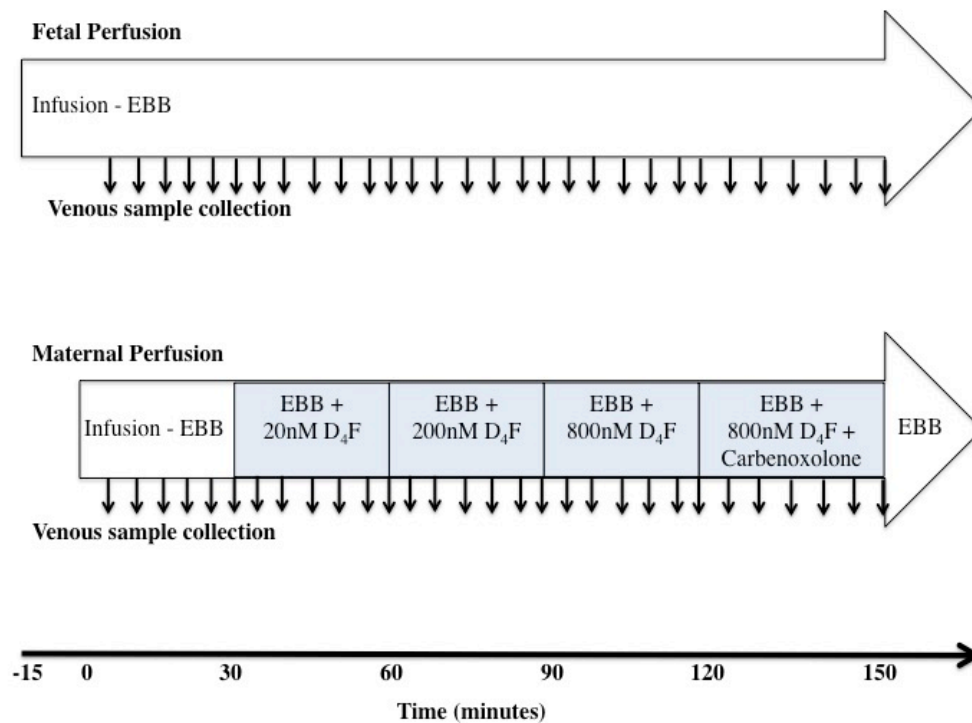


Figure 2-4. Placental Perfusion Protocol

Maternal circulation was established fifteen minutes after fetal circulation was established and ensured > 95% venous return. Perfusion into the maternal circulation was 0-30 minutes EBB alone, 30-60 minutes EBB + 20 nM D₄-Cortisol, 60-90 minutes EBB + 200 nM D₄-Cortisol, 90-120 minutes EBB + 800 nM D₄-Cortisol, 120-150 minutes EBB + 800 nM D₄-Cortisol, 150-170 minutes EBB alone. Fetal and maternal venous outputs were sampled at 5-minute intervals.

Key: D₄F (D₄-Cortisol), EBB (Earle's Bicarbonate Buffer)

2.5 Laboratory Methods and Materials

2.5.1 ELISA

Commercially available ELISA kits were used according to manufacturers' instructions. Details of the kits and validation parameters are specified in results chapters.

Enzyme-linked immunoassay (ELISA) is a plate-based immunoassay technique used for detecting and quantifying hormones, proteins, peptides and antibodies. The ELISA kits used in this Thesis were based on the principle of competitive binding. An unknown amount of antigen (hormone of interest) present in the sample and a fixed amount of enzyme (horseradish peroxidase) labeled antigen compete for the binding sites of the antibodies coated onto the wells of a 96-well plate. After incubation the wells are washed to stop the competition reaction, and any unbound conjugate is washed off (typically, phosphate buffered saline with Tween detergent (PBS-T); wash buffer supplied with each kit was used). A detection substrate solution is then applied. The detection solution contains a molecule such as 3,3',5,5'-tetramethylbenzidine (TMB), which releases a coloured dye as a response to peroxidase enzyme activity. This forms 3,3',5,5'-tetramethylbenzidine diimine which has a blue colour. This reaction is stopped by adding 'stop solution' (sulphuric acid) which causes the blue colour to change to yellow. Following this substrate reaction, the colour intensity is inversely proportional to the amount of antigen in the sample. Spectrophotometry is used to measure light absorption at 450 nm. Results of samples can be determined directly using the standard curve.

In **Chapter 3** the following ELISA assays were used:

- Plasma Total Estradiol (Demeditec DE2693; inter-assay CV 6.72 – 9.39%)
- Plasma Total Estriol (Demeditec DE2996; inter-assay CV $\leq 10\%$)

- Plasma Total Progesterone (Demeditec DE 1561; inter-assay CV 5.4 – 6.99%)
- Plasma Sex-Hormone-Binding-Globulin (Demeditec DE 2996; inter-assay CV 3.1 – 8.0%)
- Saliva Cortisol (Salimetrics, 1-3002; inter-assay CV 3 – 11%)

The ELISA for measurements of CBG were performed by John Lewis at the Canterbury Health Laboratories in New Zealand, using a previously reported method (Lewis and Elder, 2013, Kassebaum et al.). Measurements included ‘total’ CBG and ‘intact’ CBG (which has binding affinity for cortisol). ‘Cleaved’ CBG (which lacks binding affinity for cortisol) was then calculated as a ratio of total:intact CBG.

In **Chapter 5** the following ELISA assays were used:

- Plasma Adenocorticotrophic Hormone (Demeditec DE3647; inter-assay CV 6.9 – 7.1%)
- Saliva cortisol (IBL, RE52611; inter-assay CV 4.2 – 17.0%)*

Interstitial fluid cortisol levels were determined using saliva cortisol ELISA (IBL, RE52611)* using the method reported by (Bhake et al., 2013). Free cortisol values were expected to be above the highest standard, therefore were diluted to 1:10 in the assay zero standard and results were corrected for dilution.

2.5.3 Radioimmunoassay

Radioimmunoassay (RIA) is another technique used to measure concentrations of ‘antigens’ (such as hormones) by use of antibodies. To perform an RIA, a known quantity of the antigen (hormone of interest) is labeled with a radioactive isotope of iodine (such as I^{125}). The biological sample (eg. serum), containing an

unknown quantity of the same antigen is then added to the plate well. Radiolabeled and unlabeled antigens then compete for antibody binding sites on the plate. At increasing concentrations of unlabeled antigen, more of it binds to the antibody, thus preventing the radiolabeled antigen from binding and reducing the ratio of antibody-bound radiolabeled antigen to free radiolabeled antigen. The unbound and bound antigens are separated, and radioactivity of the bound antigen is measured using a gamma counter. Results of samples can be determined by reading from a standard 'binding curve'.

In **Chapter 3** total serum cortisol was measured by RIA by Graham Harold (Senior Technician) using ImmunChem Cortisol I¹²⁵;ICN Biomedicals 07221102. Corticotrophin releasing hormone was measured in plasma by extracted radioimmunoassay by Maria Bowman and Roger Smith at the University of Newcastle, Australia, using a previously reported method (Smith R, 2009).

2.5.4 Liquid- and Gas-Chromatography Tandem Mass Spectrometry

Mass spectrometry is regarded as the 'gold standard' for quantifying steroid concentrations (Stanczyk and Clarke, 2010). The advantage of this technique over immunoassays is that it can precisely measure multiple analytes at low concentration concomitantly, from a small sample volume. Chromatography is employed prior to mass spectrometry to separate analytes with different molecular masses in a mixture on the basis of their elution times as they pass along a column (**Figure 2-5**).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a technique where a sample mixture is separated by liquid chromatography prior to being ionized and characterized by mass to charge ratio and relative abundance using two mass spectrometers in series. This method can analyse biochemical, organic and inorganic compounds. The sample is forced by a liquid at high pressure

(mobile phase) through a column packed with irregular or spherical particles (stationary phase) that accomplish separation of the sample. Interactions between the different analytes and the stationary phase influence movements of the compounds in the mixture through the column, so that they are eluted at different times depending on their molecular mass.

Gas chromatography tandem mass spectrometry (GC-MS/MS) follows similar principles but uses an inert gas for the mobile phase (such as helium), and a thin layer of liquid for the stationary phase.

Tandem mass spectrometry (MS/MS) consists of several steps of mass spectrometry selection between which some form of fragmentation occurs. In the first stage of mass spectrometry, ions are formed and then separated by mass to charge ratio. Ions of a particular mass to charge ratio (known as precursor ions) are selected and fragment ions (known as product ions) are created by a collision-induced dissociation. The ions that result are separated and detected in the second stage of mass spectrometry.

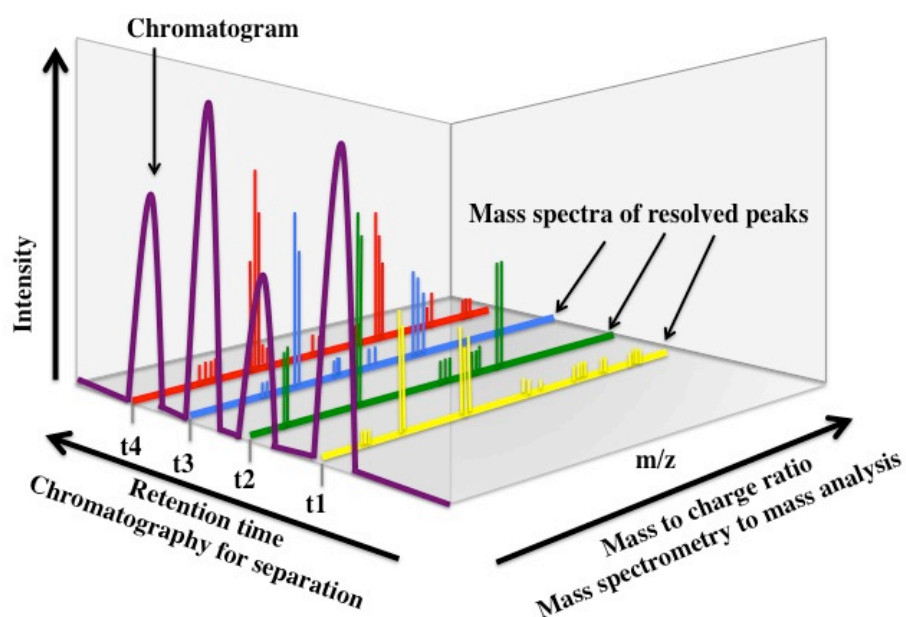


Figure 2-5 Chromatography and Mass Spectrometry

Key: t (retention time), m/z (mass to charge ratio).

In **Chapter 3** urine glucocorticoid metabolites from 24-hour urine collections were extracted by solid phase extraction and hydrolysis of conjugates and formation of their methoxime-trimethylsilyl derivatives, as previously described (Stimson et al., 2014). Analytes were quantified by GC-MS/MS spectrometry, as previously described (Andrew et al., 1998). Extraction and mass spectral analysis was performed by Alison Rutter (Research Technician).

In **Chapters 4-6**, glucocorticoids were quantified by LC-MS/MS. In **Chapter 4** maternal and cord glucocorticoids (cortisol, cortisone, corticosterone and 11-

dehydrocorticosterone) were extracted from plasma by liquid-liquid extraction, by George Just (Mass Spectrometry Core Lab Manager). In **Chapter 5**, maternal glucocorticoids (cortisol, cortisone, corticosterone and 11-dehydrocorticosterone) were extracted from serum via supported liquid extraction (SLE). In **Chapter 6**, endogenous glucocorticoids (cortisol, cortisone, corticosterone and 11-dehydrocorticosterone) and exogenous tracer glucocorticoids (D4-Cortisol, D3-Cortisone and D3-Cortisol) were extracted from placental tissue and perfusate via solid-phase extraction. Details of the extraction procedures and mass spectral conditions are included in the relevant chapters.

2.5.5 Coolens' Equation

In **Chapter 3**, estimated free cortisol was calculated using Coolens' equation (Coolens et al., 1987), which uses total cortisol and CBG levels. Unbound cortisol is represented by U (μM), molar concentration of total cortisol in by C (μM), proportion of albumin-bound cortisol by N , and the affinity of CBG for cortisol at 37 °C by K . As precise albumin levels were unavailable, a value for K can be assumed as $3 \times 10^{-7} \text{ M}^{-1}$, and a value for N to be 1.74. The following equation can be used:

$$U = \sqrt{Z^2 + 0.0122C} - Z$$

Where $Z = 0.0167 + 0.182 (T-C) \mu\text{M}$.

2.6 Materials

2.6.1 General Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma.

Item	Supplier
ACTH ELISA Estradiol ELISA Estriol ELISA Progesterone ELISA Sex hormone binding globulin ELISA	Demeditec
Saliva cortisol ELISA (saliva)	Salimetrics
Saliva cortisol ELISA (Interstitial fluid)	IBL
Cortisol RIA	DIASource
HPLC Grade Acetonitrile Ethanol Methanol Isopropanol Dichloromethane	VWR Chemicals, Boh Prolabo
HPLC Grade Water	Fisher Chemicals
Epi-cortisol Epi-corticosterone Cortisol Cortisone Corticosterone 11-dehydrocorticosterone	Steraloids
D4-cortisol	QMX Laboratories
D3-cortisol	Cambridge Isotope Laboratories
Calcium chloride + 2H ₂ O Sodium phosphate dibasic dehydrate	BDH Analytical Chemicals
Sodium heparin	Fannin

2.4.2 Equipment

Equipment	Manufacturer
MTS 4 Microtitre Shaker	IKA®
Stuart ® Roller Mixer SRt6	Cole Palmer
LabSystems Multiskan EX Original Plate Reader	ThermoFisher Scientific
Heraeus™ Megafuge™ 16 Centrifuge Series	ThermoFisher Scientific
LKB Wallax 1261 Multigamma Gamma Counter	Pharmacia
SPE Dry 96 Dual, Argonaut	Biotage
Dri-Block DB 3A	Techne
IST VacMaster 96	Biotage
Collection device tubing	Cole Parmer
3M Tegaderm Dressing	Bunzl Healthcare
MD 66 Linear Catheter T1 Perfusion Fluid Pump Syringe Pump Battery MD 107 Pump	Linton
Red Tubing Adapters FEP Tubing MAB 8 Connector	Royem Scientific
IP300 Printer	Brady®
Blunt needles (14G, 19G)	OctoInkjet
Polysorb 4-0 Suture	Coviden
Surgical instruments	Sigma
Parafilm ®	Bemis Na
Waterbath	Grant
Peristaltic pump	Watson Marlow
Peristaltic pump tubing	Elkway
2mm tubing	Portex ®
Gas 95% air + 5% CO ₂ 95% N ₂ + 5% CO ₂	BOC
Pressure Transducer	Mescap ®
Incubator	Hybaid

2.4.3 Software

Item	Supplier
MasterPlex® Readerfit	MairiBio Hitachie Solutions America, Ltd
Multicalc Immunoassay Data Manager Program	Pharmacia
IdentiLab	Brady®
SPSS v21	IBM
Prism v7.0	GraphPad
Analyst® Software 1.6.2 MultiQuant	ABSciex

2.4.4 Buffer Solution

The following solution was prepared with Milli-Q water in **Chapter 6**.

Earle's Bicarbonate Buffer (EBB): 5 mmol l⁻¹ glucose, 1.8 mmol l⁻¹ CaCl₂, 0.4 mmol l⁻¹ MgSO₄, 116.4 mmol l⁻¹ NaCl, 5.4 mmol l⁻¹ KCl, 26.2 mmol l⁻¹, NaHCO₃, 0.9 mmol l⁻¹ NaH₂PO₄

2.6 Statistical Analysis

Normal distribution of data was assessed visually using histograms. Data that were not normally distributed were normalized with the natural log transformation. Correlations of normally distributed variables were analysed using the Pearson correlation.

Adjustments for confounding factors (specified in text when describing relevant analysis) were performed using linear regression. Details of statistical methods used for each experimental paradigm are detailed in the results chapters (**Chapters 3-6**).

Data in text are presented as mean (\pm SD), and data on figures are presented as mean (\pm SEM). Statistical significant was considered at $p < 0.05$. Analysis was performed using SPSS v21 (IBM, New York, USA) and figures were prepared using GraphPad Prism v7.0 (GraphPad Software Inc, California, USA).

Chapter 3

Decreased Maternal Hypothalamic-Pituitary-Adrenal Axis Activity in Very Severely Obese Pregnancy: associations with birthweight and gestation at delivery

The following materials have been published in *Psychoneuroendocrinology* in 2016 (Stirrat et al., 2016) under the same title by Laura I Stirrat (LS), Dr James R O'Reilly (JR), Dr Sarah M Barr (SB), Professor Ruth Andrew (RA), Dr Simon C Riley (SR), Dr Alexander F Howie (AF), Maria Bowman (MB), Professor Roger Smith (RS), Dr John G Lewis (JL), Dr Fiona C Denison (FD), Dr Shareen Forbes (SF), Professor Jonathan R Seckl (JS), Professor Brian R Walker (BW), Professor Jane E Norman (JN) and Professor Rebecca M Reynolds (RR). All participants were cared for by RR, FD, SF and JN. LS conducted laboratory analyses of cortisol estradiol, estriol, progesterone, sex hormone binding globulin (SHBG), collated and analysed all data. JR conducted cortisol laboratory analyses. MB and RS conducted CRH assays. JL conducted CBG assays. SB conducted the study from which urine samples were obtained. LS prepared the first draft of the manuscript under the guidance of RR. All authors provided critical insight for the manuscript.

In summary, this work demonstrated that severely obese pregnant women had significantly lower levels of circulating cortisol, CRH, CBG, estradiol, progesterone and SHBG compared with lean pregnant women. Total urinary glucocorticoid metabolites increased significantly in lean pregnancy, but not in obese. Lower maternal cortisol in obese tended to be associated with increased birthweight, and lower CRH was a significant predictor of longer gestational

length. This work concluded that lower maternal cortisol without an increase in urinary glucocorticoid clearance may indicate a lesser activation of the HPA axis than in lean pregnancy and that this may offer a novel mechanism underlying increased birthweight and longer gestation in obese pregnancy.

3.1 Abstract

Background: The maternal HPA axis undergoes dramatic activation during pregnancy. Increased cortisol and CRH associate with low birthweight and preterm labour. In non-pregnant obesity, the HPA axis is activated but circulating cortisol levels are normal or lower than in lean women. We hypothesized that maternal cortisol levels would be lower in obese pregnancy, and would associate with increased fetal size and gestation at delivery.

Method: Fasting serum cortisol was measured at 16, 28 and 36 weeks gestation and at 3-6 months postpartum in 276 severely obese and 135 lean women. In a subset of obese (n=20) and lean (n=20) we measured CRH, hormones that regulate bioavailable cortisol (corticosteroid-binding-globulin, estradiol, estriol, progesterone). Urinary glucocorticoid metabolites were measured in pregnant (obese n=6, lean n=5) and non-pregnant (obese n=7, lean n=7) subjects.

Results: Maternal cortisol and HPA axis hormones were lower in obese pregnancy. Total urinary glucocorticoid metabolites increased significantly in lean pregnancy, but not in obese. Lower maternal cortisol in obese tended to be associated with increased birthweight ($r=-0.13$, $p=0.066$). In obese, CRH at 28 weeks correlated inversely with gestational length ($r=-0.49$, $p=0.04$), and independently predicted gestational length after adjustment for confounding factors (mean decrease in CRH of -0.25 pmol/L (95% CI -0.45 to -0.043 pmol/L) per day increase in gestation).

Conclusion: In obese pregnancy, lower maternal cortisol without an increase in urinary glucocorticoid clearance may indicate a lesser activation of the HPA axis than in lean pregnancy. This may offer a novel mechanism underlying increased birthweight and longer gestation in obese pregnancy.

3.2 Introduction

There has been a global rise in obesity in the last three decades (Ng et al., 2014). Around 64% of women of reproductive age in the United States are overweight and 35% are obese (Flegal et al., 2012) with a similar pattern in Europe (Heslehurst et al., 2010, Fitzsimons et al., 2010). There is a strong temporal gradient of increasing risk of adverse outcomes with increasing maternal body mass index (BMI; kg/m²) (Denison et al., 2008, Denison et al., 2014) including increased birthweight (Ehrenberg et al., 2004) with associated cardiovascular disease in adulthood (Ornøy, 2011), prolonged pregnancy (Caughey et al., 2009) and stillbirth (Chu et al., 2007). Mechanisms underlying the links between maternal obesity and adverse pregnancy outcomes are unknown.

Glucocorticoids are vital for fetal growth and organ maturation, but fetal over-exposure to cortisol is associated with intrauterine growth restriction (Stewart et al., 1995), and increased cardiovascular risk in later life (Reynolds, 2013). During pregnancy, the maternal HPA axis is activated, resulting in an exponential increase in the maternal circulating levels of cortisol and CRH (Duthie and Reynolds, 2013). A number of endocrine changes contribute to the increase in total cortisol levels. The placenta releases CRH from the second trimester (~16 weeks gestation) onwards leading to a complex feed-forward loop, with placental CRH activating the maternal HPA axis, which generates cortisol, and maternal cortisol simultaneously stimulates placental CRH synthesis, thus further increasing cortisol levels (Jung et al., 2011). Increased hepatic synthesis of CBG (the predominant binding protein of cortisol) during pregnancy, driven by estrogen stimulation (Mazer, 2004), is considered to contribute to the rise in maternal total cortisol (Jung et al., 2011). Rising levels of progesterone, which displaces cortisol from CBG, may also contribute to excess of free, bioavailable cortisol (MacLaughlin et al., 1972). Pregnancy is associated with increased cortisol clearance (via hepatic metabolism and urinary excretion), suggesting that in addition to elevated CRH and CBG, an overall increase in maternal HPA axis

activity contributes to the increase in total maternal cortisol with advancing gestation (Jung et al., 2011). The HPA axis is thought to play a key role in governing the length of gestation and triggering the onset of parturition. High maternal circulating CRH at 31-33 weeks gestation is associated with preterm labour (< 37 weeks), and low levels with post-term pregnancy (> 41 weeks) (Sandman et al., 2006, Wadhwa et al., 2004). It is not known whether maternal CRH is altered in obese pregnancy, or whether these alterations influence the duration of pregnancy.

Non-pregnant obesity is also associated with activation of the HPA axis, and also with increased clearance of cortisol (Marin et al., 1992) leading to normal or lower levels of total circulating cortisol (Praveen et al., 2011). Further, increased BMI has been negatively correlated with awakening cortisol levels (Champaneri et al., 2013). Preliminary evidence from two recent studies of obese pregnant women suggests that the HPA axis changes in non-pregnant obesity may be maintained during obese pregnancy. Obese women ($\text{BMI} \geq 30 \text{ kg/m}^2$) in early pregnancy were found to have lower morning salivary cortisol (Aubuchon-Endsley et al., 2014), and lower total cortisol (Luiza et al., 2015) than pregnant women with a normal BMI.

To our knowledge, there are no reports of HPA axis hormones and their association with clinical outcomes in very severely obese ($\text{BMI} \geq 40 \text{ kg/m}^2$) pregnant women. We therefore aimed to measure total morning cortisol levels in very severely obese (grade 3 obesity, $\text{BMI} \geq 40 \text{ kg/m}^2$) pregnant women and a lean control group ($\text{BMI} < 25 \text{ kg/m}^2$). In sub-studies we further characterized the HPA axis in obese pregnancy to identify potential underlying mechanisms for the predicted lower maternal cortisol in obese pregnancy. We hypothesized that maternal CRH and factors that may reduce (CBG, estradiol) or increase (progesterone) free cortisol levels would be altered in obese pregnancy, in favour of lowering maternal cortisol, and that urinary glucocorticoid metabolites would be increased in obese pregnant women, in-keeping with increased HPA axis

activity, similar to non-pregnant obesity. Finally to test the hypothesis that reduced fetal glucocorticoid exposure in obese pregnancy would contribute to higher birthweight and longer gestation we explored associations with clinical outcomes related to fetal growth and pregnancy duration.

3.3 Methods

3.3.1 Participants

We recruited 286 severely obese and 137 lean pregnant women with singleton pregnancies to a longitudinal study of obesity in pregnancy (Hormones in Pregnancy; HIP). Eligible women had a singleton pregnancy and a normal booking ultrasound scan. Women who used regular glucocorticoid medication during pregnancy (obese $n=7$, lean $n=1$) or received antenatal steroids for suspected preterm delivery were excluded (obese $n=3$, lean $n=1$), resulting in a final dataset of 276 obese and 135 lean women. Ethical approval and written informed consent were obtained. Participants reported demographic information on questionnaires and attended for study visits at 16 weeks, 28 weeks, and 36 weeks gestation and between 3 and 6 months postpartum. At each study visit, fasting morning blood samples were obtained between 08.30h and 09.30h. At the first study visit, a subset of women (obese $n=14$, lean $n=38$) collected three saliva samples for analysis of cortisol, to assess the cortisol awakening response, using a Salivette® collector (Sarstedt, Numbrecht, Germany); samples were collected at bedtime, at waking, and 30-minutes after waking, and were stored at -80°C until analysis.

A separate cohort of women provided 24-hour urine collections for analysis of glucocorticoid metabolites. Obese and lean pregnant women provided collections at 19 weeks and 36 weeks gestation, and non-pregnant obese and lean controls provided one collection (obese pregnant $n=6$, obese non-pregnant $n=7$, lean

pregnant n=5, lean non-pregnant n=7). Groups are referred to as LNP (lean non-pregnant), ONP (obese non-pregnant), LP1 (lean pregnant visit 1, approximately 19 weeks gestation), LP2 (lean pregnant visit 2, approximately 36 weeks gestation), OP1 (obese pregnant visit 1, approximately 19 weeks gestation) and OP2 (obese pregnant visit 2, approximately 36 weeks gestation). Total urine volume was recorded and stored in 20 mL aliquots at -80 °C until analysis. Eligible women were aged between 18-45 years and were Caucasian. Exclusion criteria were diabetes, active endocrine disorders, use of glucocorticoid medications or multiple pregnancy.

Clinical outcomes were extracted from medical records. Macrosomia was defined as a birthweight ≥ 4000 g at term (≥ 37 weeks). Low birthweight was defined as ≤ 2500 g.

3.3.2 Laboratory Methods

Commercially available radioimmunoassay kits were used for the analysis of total serum cortisol (ImmuChem Cortisol¹²⁵ I; ICN Biomedicals, CA, USA; inter-assay CV 7.6%). We measured CBG, CRH, estradiol, estriol, progesterone and SHBG in plasma samples from a subset of the HIP study cohort (obese n=20, lean n=20). CBG was measured by ELISA as described by Lewis et al., (Lewis and Elder, 2013); this included ‘total’ CBG and ‘intact’ CBG (which has binding affinity for cortisol). ‘Cleaved’ CBG (which lacks binding affinity for cortisol) was then calculated as a ratio of total:intact CBG. Free cortisol was calculated when both cortisol and CBG measurements were available using Coolens’ equation (Coolens et al., 1987), which uses total cortisol and CBG levels. CRH was measured by extracted radioimmunoassay as described by Smith et al., (Smith R, 2009). ELISA kits were used to measure total estradiol (Demeditec DE2693; inter-assay CV 6.72-9.39%), total estriol (Demeditec DE3717; inter-

assay CV $\leq 10\%$) total progesterone (Demeditec DE1561; inter-assay CV 5.4-6.99%), and SHBG (Demeditec DE2996; inter-assay CV 3.1-8.0%).

Urine glucocorticoid metabolites were quantified in 24-hour urine collections by gas chromatography electron impact tandem mass spectrometry following solid phase extraction hydrolysis of conjugates and formation of their methoxime-trimethylsilyl derivatives, as previously described (Stimson et al., 2014). Metabolites included urinary free cortisol and its metabolites (5β -tetrahydrocortisol (THF), 5α -tetrahydrocortisol (α -THF), α -cortol, and β -cortol); and urinary cortisone and its metabolites (tetrahydrocortisone (THE), α -cortolone, β -cortolone). We measured the sum of total glucocorticoid metabolites (THF, α -THF, THE, α -cortol, β -cortol, α -cortolone, β -cortolone), as a measure of total cortisol production. The ratio of cortisol:cortisone were examined as a marker of 11beta-hydroxysteroid-dehydrogenase-type 2 (11β -HSD2) enzyme activity, which converts active cortisol into inactive cortisone; the ratio of α -THF:cortisol as a measure of 5α -reductase activity, which metabolises cortisol to α -THF; and the ratio of (THF+ α -THF):THE as an indicator of the balance of whole-body 11beta-hydroxysteroid dehydrogenase enzyme activities (11β -HSD1 and 11β -HSD2 activity (Andrew et al., 1998).

3.3.3 Statistical Analysis

Data distribution was assessed for normality by visually assessing histograms. Data that were not normally distributed were normalized using the natural-log transformation. The independent t-test was used to test for differences between continuous variables and the chi-squared test for categorical variables. Repeated measures analysis of variance (ANOVA) was used to compare the pattern of change in hormones with advancing gestation between obese and lean. Correlations between HPA axis hormones with birthweight and gestation at

delivery were tested and regression analysis used to adjust for confounding factors, including smoking status, baby gender, and gestational age at delivery. Analysis was performed using SPSS v20 (IBM). Data in text are mean \pm (sd), and data in figures are mean \pm SEM. Statistical significance was considered at $p < 0.05$.

Power calculations: A formal sample size calculation was not performed for either cohort. We aimed to generate data for over 200 very severely obese and 100 lean control women for the HIP study. Post-hoc power calculations using G*Power3.1 software (Faul et al., 2009) showed that a sample size of $n=462$ was required for 80% power to demonstrate an inverse correlation between cortisol and birthweight at the 5% significance level.

3.4 Results

3.4.1 Demographics

Table 3-1 shows characteristics of the 276 obese and 135 lean women and their babies. Obese women were younger than lean, had greater social deprivation (P, 2004), were of higher parity and were more likely to smoke. **Appendix Table 3-1** shows characteristics of women included in the subset of obese ($n=20$) and lean ($n=20$) for analysis of CBG and sex steroids; and **Appendix Table 3-2** shows characteristics of women who provided 24-hour urine collections.

MATERNAL CHARACTERISTICS	Obese (n=276)	Lean (n=135)	p-Value
Age (years)	31.2 (5.3)	33.0 (4.6)	<0.001
BMI (kg/m²)	44.0 (4.0)	22.7 (1.7)	< 0.001
Parity n (%) - Prim - Para 1 - Para ≥ 2	137 (49.6) 82 (29.7) 57 (20.7)	84 (62.2) 41 (30.4) 10 (7.4)	0.002
Ethnicity n (%) - Caucasian - Non-Caucasian	263 (95.3) 13 (4.7)	133 (98.5) 2 (1.5)	0.10
DepCat Score n (%) - 1-3 - 4+	80 (29.0) 196 (71.0)	90 (66.7) 45 (33.3)	<0.001
Smoking Status n (%) - Never Smoked - Ex-smoker - Current smoker	161 (58.8) 79 (28.8) 34 (12.4)	81 (60.0) 50 (37.0) 4 (3.0)	0.005
Labour onset Mode n (%) - Spontaneous - Induced - No labour	111 (40.2) 105 (38.0) 56 (20.3)	96 (71.1) 30 (22.2) 9 (66.7)	<0.0001
Mode of Delivery n (%) - Spontaneous vertex delivery - Assisted vaginal delivery - Emergency Caesarean section - Elective Caesarean section	121 (44.5) 36 (13.2) 67 (24.5) 49 (17.8)	75 (55.5) 31 (23.0) 21 (15.6) 8 (5.9)	<0.0001
Medical Complications n (%) - Gestational diabetes - Pre-eclampsia - Pregnancy induced hypertension	43 (15.6) 5 (1.8) 14 (5.1)	2 (1.5) 3 (2.2) 2 (1.5)	<0.0001 0.80 0.07
TERM BABIES (≥ 37 weeks)	Obese (n=262)	Lean (n=132)	p-value
Baby Sex - Male n (%) - Female n (%)	123 (46.9) 139 (53.1)	63 (47.7) 69 (52.3)	0.88
Gestational age at delivery (days)	280.4 (8.6)	282.5 (8.3)	0.02
Birthweight (g)	3629.1 (531.8)	3571.6 (498.4)	0.30
Macrosomia (>4000g) - Yes n (%) - No n (%)	64 (24.4) 198 (75.6)	25 (18.9) 107 (81.1)	0.22
Low birth weight (<2500g) - Yes n (%)	4 (1.5)	0 (0)	0.15
Weight at 3months of age (kg)	3583.0 (580.6)	3554.0 (507.6)	0.62
PRETERM BABIES (< 37 weeks)	Obese (n=14)	Lean (n=3)	p-value
Birthweight (g)	2721.1 (780.6)	2776.7 (257.9)	0.91
Gender n (%) - Male - Female	10 (71.4) 4 (28.6)	1 (33.3) 2 (66.7)	0.21
Gestational age at delivery (days)	250.4 (14.5)	256.3 (1.5)	0.91

Table 3-1. Maternal and Neonatal Characteristics

Table 3-1. (cont.)

Parity was defined as ‘prim’ (no previous pregnancies delivered after 24 weeks), ‘para 1’ (one previous delivery after 24 weeks gestation) and ‘para ≥ 2 ’ (two or more previous deliveries after 24 weeks gestation). Socioeconomic status was measured using deprivation category (DepCat) scores based on postcode (P, 2004). DepCat score 1-3 represents the least deprived; score 4+ represents the most deprived. DepCat information was unavailable for 1 obese woman. ‘Smokers’ were defined as those who considered themselves as current smokers. Term babies characteristics excludes those who were exposed to antenatal glucocorticoids. Missing data: smoking status (obese n=2), labour onset mode (obese n=4), mode of delivery (obese n=3). Data are mean (sd) or N (%).

3.4.2 Gestational Changes in HPA Axis Hormones

3.4.2.1 Cortisol and CBG Levels are Lower in Obese Pregnancy

Total serum cortisol concentrations were higher in pregnancy than in non-pregnancy in obese and lean women but were significantly lower in obese than in lean at each time-point during pregnancy and postpartum (**Figure 3-1a**). The diurnal pattern of cortisol measured from saliva cortisol (peak cortisol levels on waking) was maintained in both obese and lean women (**Figure 3-1b**). Obese women had apparent ‘blunting’ of the cortisol awakening response with significantly lower cortisol levels in samples collected at 30-minutes after waking than in lean women. Bedtime and waking saliva cortisol did not differ between obese and lean. Mean total cortisol at 16 weeks gestation did not differ according to maternal age, social class, parity or smoking status during pregnancy (data not shown). There were no differences in cortisol levels at any time according to gestational diabetes or pre-eclampsia status (data not shown).

Total and intact CBG rose similarly throughout pregnancy in both lean and obese women (**Figure 3-2**). Total CBG levels were significantly lower in obese compared with lean women at 28 weeks and postpartum. Intact CBG did not differ between obese and lean at any time-point, and cleaved CBG was significantly lower in obese postpartum. Free cortisol levels calculated using intact CBG values were significantly lower in obese than in lean at 28 and 36 weeks, while free cortisol calculated using total CBG was lower in obese compared with lean at 36 weeks (**Table 3-2**). Although absolute levels of free cortisol calculated with intact CBG levels appeared higher than levels calculated using total CBG values, this was not statistically significant.

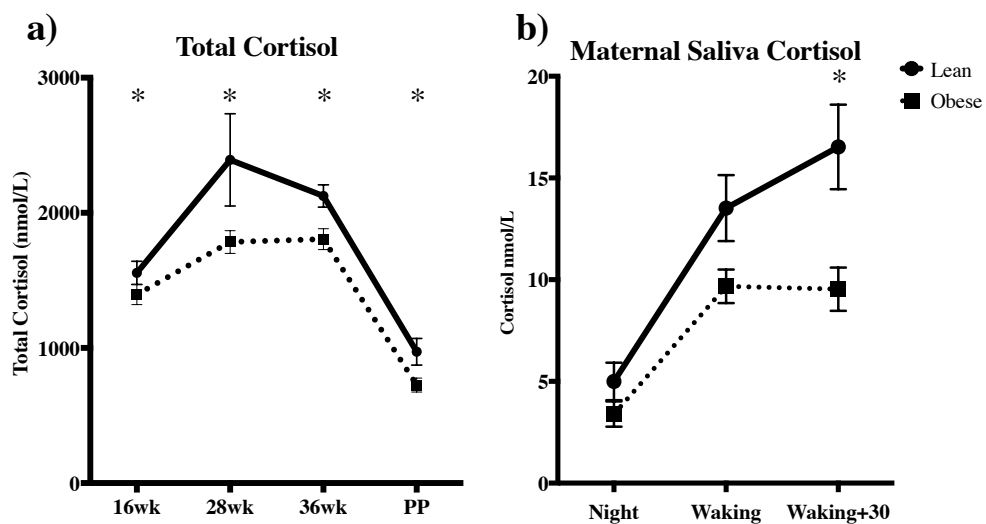


Figure 3-1. Total Serum Cortisol (3-1a) and Saliva Cortisol (3-1b)

Total serum cortisol was significantly lower in obese than lean at 16 weeks ($p=0.02$), 28 weeks ($p=0.003$), 36 weeks ($p=0.001$) and postnatally ($p=0.01$). Pattern of cortisol change in pregnancy was similar in obese and lean ($p=0.79$). Diurnal rhythm was maintained in saliva cortisol profiles similarly in obese and lean ($p=0.94$), with significantly lower levels in obese at 30 minutes after waking

($p=0.02$). Data are mean (sem); * $p<0.05$, ** $p\leq 0.01$, *** $p\leq 0.0001$. Sample size obese ($n=276$), lean ($n=135$).

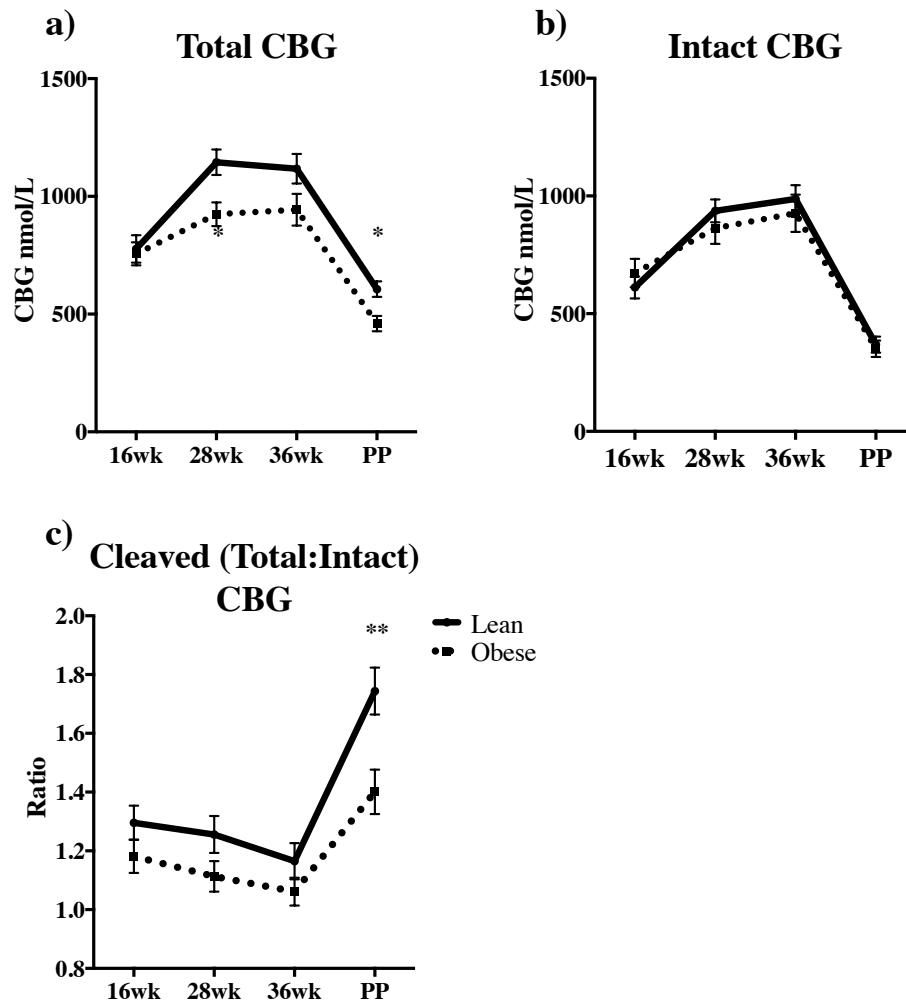


Figure 3-2a-c. Total corticosteroid binding globulin (CBG) (3-2a), Intact CBG (3-2b) and Cleaved CBG (3-2c)

The patterns of change throughout pregnancy were similar in obese and lean for total CBG ($p=0.66$), intact CBG ($p=0.35$) and cleaved CBG ($p=0.64$). Total CBG was significantly lower in obese at 28 weeks ($p=0.005$) and postnatal ($p=0.003$). Cleaved CBG was significantly lower postpartum ($p=0.003$). Intact CBG did not

differ significantly between obese and lean. Data are mean (sem); * $p < 0.05$, ** $p \leq 0.01$. Samples size obese (n=20), lean (n=20).

Free Cortisol (nmol/L)	Obese (n=20)	Lean (n=20)	p-Value
Calculated with Total CBG			
16 weeks	482.6 (484.1)	473.9 (342.8)	0.63
28 weeks	392.4 (225.9)	431.4 (156.5)	0.32
36 weeks	326.1 (177.9)	617.0 (330.0)	0.001
Postpartum	293.6 (180.2)	329.8 (211.5)	0.628
Calculated with Intact CBG			
16 weeks	552.8 (521.0)	729.4 (337.3)	0.22
28 weeks	403.2 (227.8)	508.9 (123.7)	0.005
36 weeks	349.6 (164.1)	646.8 (324.4)	0.003
Postpartum	314.0 (205.3)	370.6 (208.8)	0.403

Table 3-2 Free Cortisol calculated using total and intact CBG levels.

Data are mean (sd).

3.4.2.2 In Obese Pregnancy, Hormones Regulating Cortisol Levels are Lower

CRH concentrations rose throughout pregnancy. The greatest increase was between 28 and 36 weeks, but the rise was less marked in obese women (**Figure 3-3a**). CRH levels were significantly lower in obese women at 28 weeks (obese 42.2 ± 26.7 pmol/L, lean 25.2 ± 17.1 pmol/L; $p=0.02$) with a similar trend at 36 weeks (obese 316.1 ± 244.4 pmol/L, lean 193.7 ± 141.1 pmol/L; $p=0.06$). Estradiol, estriol and progesterone concentrations all rose throughout pregnancy, but the rise was less marked in obese women (**Figure 3-3b**). Estradiol (**Figure 3-**

3b) and estriol (**Figure 3-3c**) were significantly lower in obese compared with lean at 28 and 36 weeks while progesterone (**Figure 3-3d**) was significantly lower in obese throughout pregnancy. SHBG concentrations rose throughout pregnancy, and were not different in lean and obese pregnant women during pregnancy, but were significantly lower in obese women post-partum (**Figure 3-3e**).

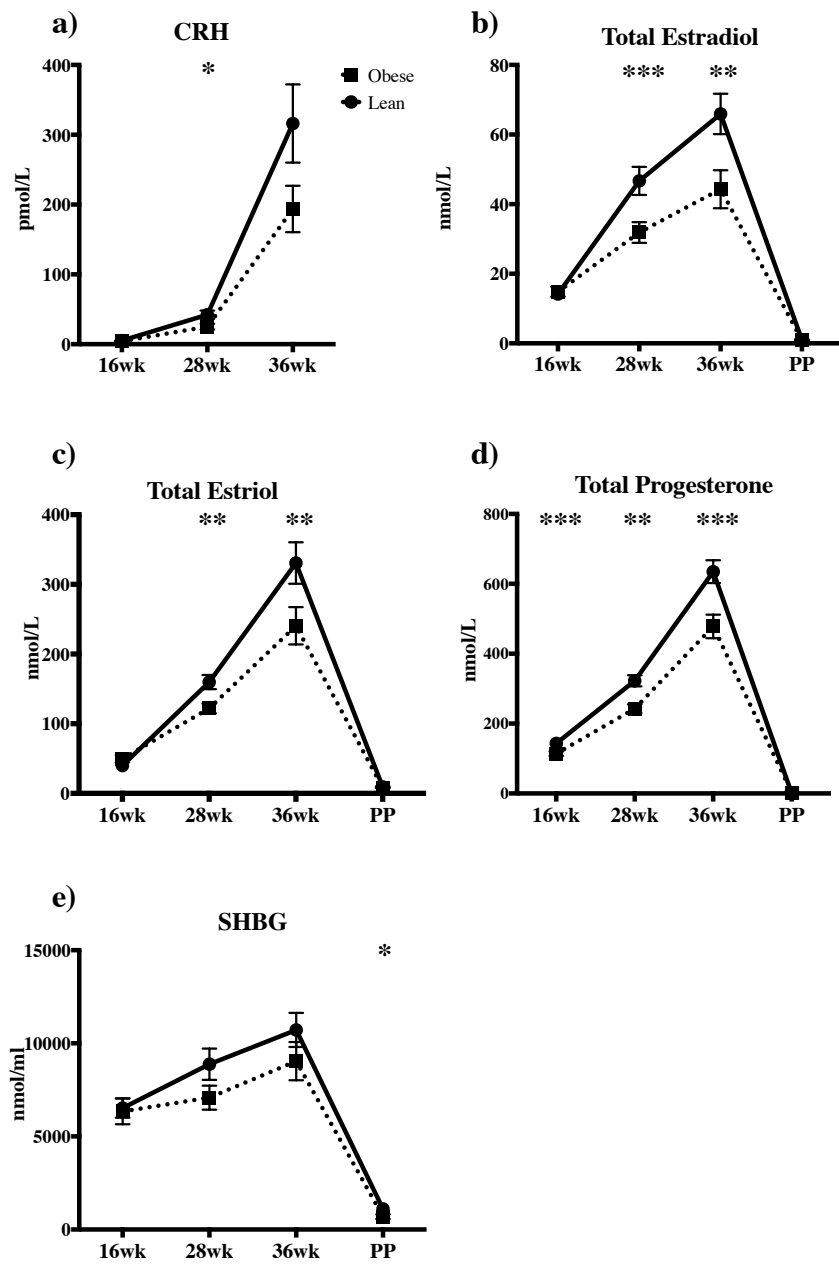


Figure 3-3a-d. Corticotrophin releasing hormone (CRH) (3a), estradiol (3b), estriol (3c) and progesterone (3d) and sex-hormone-binding-globulin (SHBG)

Figure 3-3a-d. (cont.)

The pattern of rise was significantly different between lean and obese in CRH ($p=0.025$), estradiol ($p<0.0001$), estriol ($p=0.001$) and progesterone ($p<0.0001$). CRH was significantly lower in obese at 28 weeks ($p=0.02$) with a trend at 36 weeks ($p=0.06$). Total estradiol was significantly lower in obese at 28 weeks ($p<0.0001$) and 36 weeks ($p=0.025$). Total estriol was significantly lower in obese at 28 weeks ($p=0.002$) and 36 weeks ($p=0.01$). Total progesterone was significantly lower in obese women at 16 weeks ($p=0.001$), 28 weeks ($p<0.0001$), 36 weeks ($p=0.001$) but not post-partum. ($p=0.67$). SHBG was significantly lower in obese post-partum ($p=0.019$). Data are mean (sem); * $p<0.05$, ** $p\leq 0.01$, *** $p\leq 0.0001$. Sample size obese ($n=20$), lean ($n=20$).

3.4.2.3 Urine Glucocorticoid Metabolites are not Increased in Obese Pregnancy Compared with Non-Pregnancy

Total urinary glucocorticoid metabolites were significantly higher in obese non-pregnant than in lean non-pregnant women (consistent with increased HPA axis activity and cortisol production; **Figure 3-4a**). In lean, total glucocorticoid metabolites were significantly higher at LP1 and LP2 compared to LNP. In contrast in obese, total glucocorticoid metabolites were similar during pregnancy and in non-pregnancy. There was a similar pattern of findings with urinary free cortisol (**Figure 3-4b**) free cortisone (**Figure 3-4c**), total cortisol metabolites (**Figure 3-4d**) and total cortisone metabolites (**Figure 3-4e**), with higher levels in lean pregnant compared with LNP and no significant change in obese pregnant compared to ONP.

The ratios of cortisol:cortisone (**Figure 3-4f**) and THFs:THE (**Figure 3-4g**) decreased with advancing gestation in lean pregnant compared to LNP consistent with increasing conversion of cortisol to cortisone (by whole-body 11β -HSD2), and/or decreasing regeneration of cortisol from cortisone (by 11β -HSD1). Neither ratio changed significantly during pregnancy in obese women.

The ratio of α -THF:cortisol was higher in ONP compared to LNP women (**Figure 3-4h**). In keeping with this, levels of α THF were significantly higher in ONP compared with LNP, and were higher at LP1 and LP2 compared to LNP women (data not shown). Taken together, these data suggest that metabolism of cortisol to α -THF (by 5α -reductase) is higher in LNP compared to ONP women, and that such metabolism increases in lean women with advancing gestation. The opposite pattern was observed in obese; with a lower ratio of α -THF:cortisol and lower levels of α -THF in obese pregnant compared to ONP, suggesting that metabolism of cortisol to α -THF (by 5α -reductase) is blunted in obese pregnancy.

The ratio of THE:cortisone was lower in LNP compared to ONP women, and was lower at LP1 and LP2 compared to LNP women (**Figure 3-4i**). There were no significant changes in the obese with advancing gestation. This suggests metabolism of cortisone to THE (by 5β -reductase) increases with advancing gestation in lean women, but may be blunted in obese women during pregnancy.

The ratios of cortisol:cortisone (3-4f), THFs:THE (3-4g), α THF:cortisol (3-4h) and THF:cortisone (3-4i) decreased in lean with advancing gestation, but not in obese, suggesting that activities of 11β -HSD2, 11β -HSD1, 5α -reductase and 5β -reductase are blunted in obese pregnant compared to lean pregnant women.

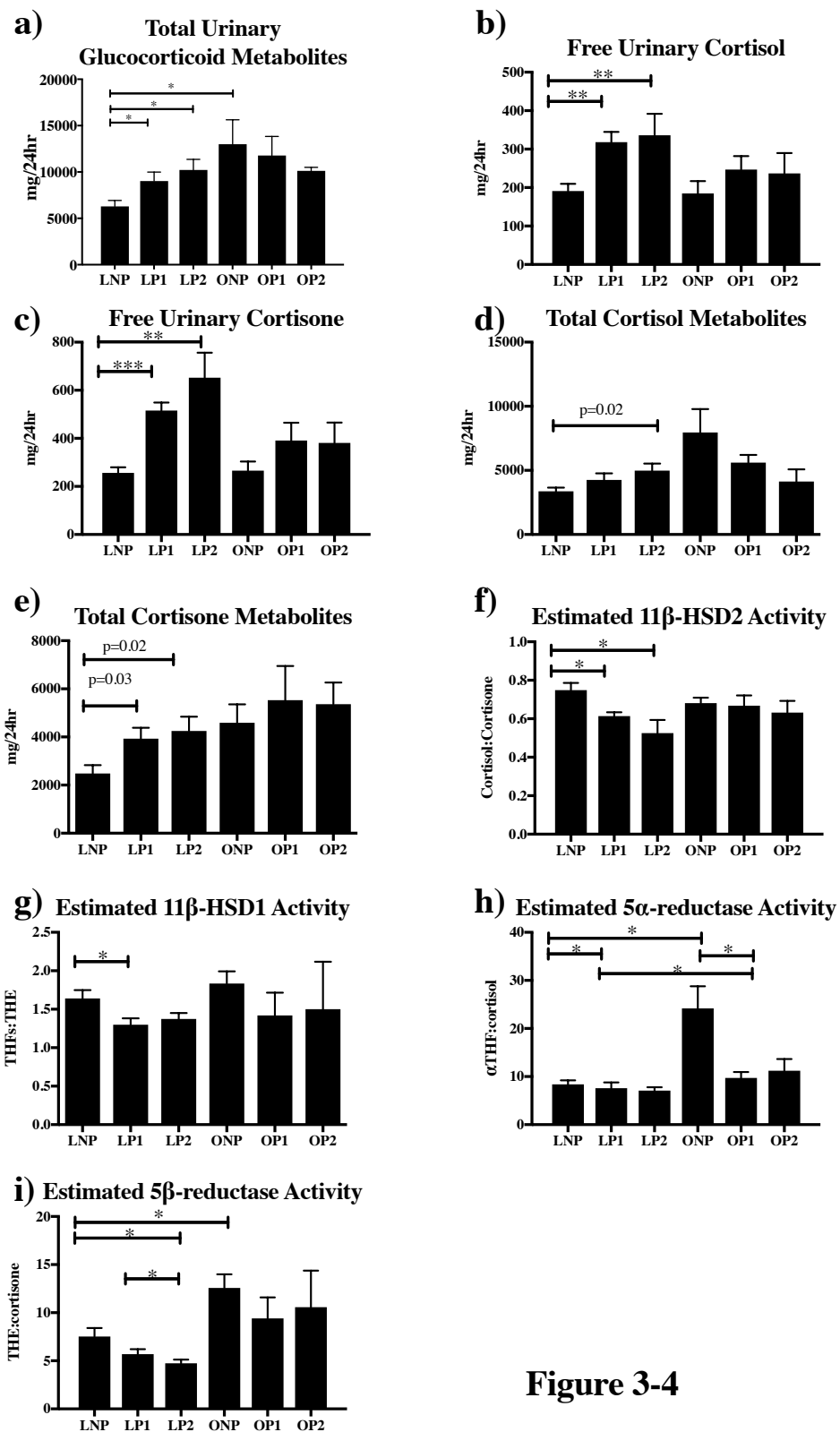


Figure 3-4

Figure 3-4a-n Urine Glucocorticoid Metabolites

(3-4a-e) Total glucocorticoid metabolites, urinary free cortisol, urinary free cortisone, total cortisol metabolites and total cortisone metabolites increased during pregnancy in lean (*), but not in obese. The ratios of cortisol:cortisone (3-4f), THFs:THE (3-4g), α THF:cortisol (3-4h) and THF:cortisone (3-4i) decreased in lean with advancing gestation, but not in obese. Data are mean (sem); * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$. Sample size LNP (n=7), ONP (n=7), LP (n=5) and OP (n=7).

Key: LNP (lean non-pregnant), ONP (obese non-pregnant), LP1 (lean pregnant visit 1, approximately 19 weeks gestation), LP2 (lean pregnant visit 2, approximately 36 weeks gestation), OP1 (obese pregnant visit 1, approximately 19 weeks gestation), OP2 (obese pregnant visit 2, approximately 36 weeks gestation), THF (5 β -tetrahydrocortisol), α -THF (5 α -tetrahydrocortisol), THE (tetrahydrocortisone), 11 β -HSD1 (11beta-hydroxysteroid-dehydrogenase-type 1) and 11 β -HSD2 (11beta-hydroxysteroid-dehydrogenase-type 2).

3.4.3 Associations of HPA Axis Hormones with Birthweight and Gestation at Delivery

Birthweight did not differ between lean and obese in the unadjusted analysis (**Table 3-1**), but was significantly higher in obese after adjustment for gestation at delivery, sex of baby, maternal age and smoking status ($\beta = 0.102$, $p = 0.03$). In lean women, increased cortisol at 16 weeks was positively associated with birthweight in unadjusted analysis though this was not significant after adjusting for smoking status, DepCat score, gestational age at delivery and baby gender

($r=0.19$, $p=0.032$, $p_a=0.10$). In contrast, in the obese there was a weak negative correlation between lower cortisol at 16 weeks and higher birthweight, though this was not significant in adjusted analyses ($r=-0.13$, $p=0.066$, $p_a=0.33$). Cortisol at 28 weeks was lower in lean women with macrosomic babies compared to appropriate for gestational age babies (1760 ± 729 nmol/L vs 2551 ± 4228 nmol/L; $p=0.048$), but did not differ in obese women with macrosomic babies, or in women with low birthweight babies. CRH did not correlate with birthweight in lean or obese.

Obese women delivered at an earlier gestation than lean women (**Table 3-1**) due to a higher proportion of elective Caesarean deliveries (obese 17.8% vs. lean 5.9%; $p<0.0001$), as there were more previous Caesarean sections (obese 49.4% vs lean 28.6%; $p=0.01$). A higher rate of medical complications in obese women meant that there was a higher rate of induction of labour before 40 weeks (obese 30.2% vs. lean 17.6%; $p<0.0001$). In women who did not undergo early iatrogenic delivery before 40 weeks the mean gestation was not significantly different between lean and obese (lean 283 ± 8 days, obese 282 ± 11 days; $p=0.53$). In obese, there was an inverse correlation between CRH at 28 weeks and gestational length ($r=-0.49$, $p=0.04$). After adjusting for maternal smoking and mode of delivery, this remained significant as an independent predictor of gestation at delivery (mean decrease in CRH of -0.25 pmol/L (95% CI -0.45 to -0.043 pmol/L) per day increase in gestation). Cortisol did not associate with gestational age at delivery in lean or obese.

3.5 Discussion

Our findings demonstrated lower fasting morning cortisol levels in very severely obese women throughout pregnancy compared with a lean control group; a pattern similar to non-pregnant severe obesity (Champaneri et al., 2013). The

lack of an increase in urinary clearance of glucocorticoids during pregnancy in obese women suggests that in contrast to non-pregnant obesity (Marin et al., 1992, Strain et al., 1980), the activation of the HPA axis, which normally occurs during pregnancy (Duthie and Reynolds, 2013, Lindsay and Nieman, 2005) was blunted in these very severely obese pregnant women. Further, the apparent ‘blunting’ of the cortisol awakening response we observed in obese pregnant women would also support the notion that obese pregnant women have lower production of cortisol. Our exploratory analysis suggests this HPA axis dysregulation may contribute to complications of obese pregnancy including higher birthweight and prolonged gestation.

Our observation that hormones regulating circulating cortisol levels including CBG, CRH and estrogens were also lower in obese pregnancy suggests these may contribute to the lower maternal cortisol in obese pregnant compared to lean pregnant women. CRH was lower in obese pregnant compared to lean pregnant women. The positive feedback loop between glucocorticoids and placental CRH (King et al., 2001) raises the question of whether lower cortisol in obese was a cause or consequence of lower CRH. Elevated maternal total cortisol at 15 weeks is predictive of a subsequent surge in placental CRH at 31 weeks in women who deliver preterm (Sandman et al., 2006). This would support lower cortisol levels in very severely obese pregnancy at an earlier gestation as the driving factor for the lower CRH we observed later in pregnancy in obese. Alternatively, if lower cortisol in obese pregnancy is a consequence of lower placental CRH; a relatively reduced drive on the maternal HPA axis (as a result of lower placental CRH) may result in lower production of ACTH from the pituitary gland, and thus lower cortisol production.

Our observations of lower progesterone and estrogen levels in very severe obese pregnancy may not be due to altered SHBG levels, which did not differ between lean and obese during pregnancy. Further, our data are consistent with recent observations in less severely obese pregnant women (Lassance et al., 2015),

where it was proposed that impaired mitochondrial function in the placentas of obese pregnant women limits placental sex steroid biosynthesis. Cortisol is not considered to be synthesised in the placenta, so it is unlikely that this contributes to the lower cortisol we found in very severely obese pregnancy. In contrast to our findings of lower cortisol and lower estrogen in very severely obese pregnant women, a small study in postmenopausal women suggested that higher estrogen levels may magnify the negative feedback of cortisol on the HPA axis in postmenopausal women, leading to lower cortisol levels (Sharma et al., 2014). To the best of our knowledge, there are no other data describing the effects of estrogen on cortisol in severely obese pregnant women. Evidence from *in vitro* studies would suggest that lower progesterone and estrogen may limit CRH production (Ni et al., 2002, Ni et al., 2004), which in turn could reduce total cortisol levels.

The measurements of total and intact CBG allowed us to calculate free, bioavailable cortisol levels, which were also lower in obese pregnant women. The reason for the lesser rise in total CBG in obese women is unknown, but likely reflects the lower estrogen levels we observed (Jung et al., 2011). Other factors that may alter CBG levels include altered metabolic, inflammatory or genetic factors. The very severely obese women in our cohort had higher levels of insulin, inflammatory markers and liver enzymes than the lean women (Forbes et al., 2015); all factors which would be anticipated to increase CBG levels. However, as we have previously demonstrated that very severely obese women failed to accumulate hepatic fat during pregnancy, we can speculate that the associated improvement in hepatic insulin sensitivity may also drive a reduction in CBG synthesis in very severely obese pregnancy. Although it has been suggested that the placenta synthesises CBG (Misao et al., 1999), whether this differs in obese and lean placental tissue is not known. Our recent observation that morning cortisol levels are genetically determined through cortisol binding to CBG (Bolton et al., 2014) implies a further mechanism for regulation of

maternal and fetal glucocorticoid exposure, which requires further exploration. Alternatively, if CRH is the primary driving force mediating cortisol levels, the lower CBG may be a mechanism by which the maternal liver balances regulation of fetal cortisol exposure and the normal physiological roles of cortisol in the mother.

The lack of rise in urinary total glucocorticoid metabolites, free cortisol, free cortisone, total cortisol metabolites and total cortisone metabolites in obese pregnant compared to lean pregnant women is consistent with decreased activity of the HPA axis in obese pregnancy, resulting in lower production of cortisol. This contrasts to the findings in the lean where total urinary glucocorticoid metabolite excretion increased with advancing gestation, paralleling the rising circulating cortisol levels, and also contrasts to non-pregnant obesity where urinary glucocorticoid clearance is increased compared to lean controls. Further, our data suggests that the changes in activity of whole-body 11β -HSD2, 11β -HSD1, 5α -reductase and 5β -reductase enzymes may be blunted in obese pregnant compared to lean pregnant women. These preliminary data would suggest that the lower circulating levels of cortisol in obese cannot be explained by increased metabolism or clearance of cortisol. Further studies are needed to explore this, and to investigate possible alternative explanations for the apparent lack of increased clearance of cortisol which has been reported in non-pregnant obesity (Marin et al., 1992).

Our data add to the emerging literature in humans that exposure to endogenous cortisol is a critical factor regulating growth and length of gestation (Duthie and Reynolds, 2013). In exploratory analyses we observed a weak inverse trend between lower cortisol at 16 weeks and higher birthweight in obese, as well as lower cortisol levels in lean women with macrosomic babies. Further, lower CRH at 28 weeks gestation in obese was also significant as an independent predictor of longer gestation. Although our study was under-powered to look at birth outcomes this is the first study to show that dysregulation of the HPA axis

may contribute to the complications of obese pregnancy including increased fetal size and prolonged gestation.

Strengths of this study include the large sample size, which allowed for repeated measures of cortisol from blood in the same individual including in pregnancy and postpartum, the careful timing of morning samples and the detailed assessment of hormones controlling cortisol release. Further, the marked differences in BMI levels between the severely obese pregnant and lean pregnant groups meant our two groups were very different for comparison. A limitation is that total cortisol was measured by immunoassay, which has been reported to detect lower levels of cortisol during pregnancy, compared with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Jung et al., 2011), however, we were still able to detect significant differences between the lean and obese study groups. Further limitations of the study are that we used Coolens' equation (Coolens et al., 1987) to estimate free cortisol levels; this has been used in other studies of cortisol in pregnancy (LeWinn et al., 2009) but not validated. The single time-point sampling would not account for variation in cortisol levels caused by hormone oscillations. Studies investigating the ultradian rhythm generated by cortisol pulsatility are on-going. Finally placental cortisol metabolism and transfer in obese pregnancy remain unknown and further studies are needed to understand more about the contribution of the placenta to maternal and fetal glucocorticoid exposure.

In conclusion, our study suggests that there is dysregulation of the HPA axis in obese pregnancy resulting in lower circulating levels of cortisol and its regulatory hormones consistent with decreased HPA axis activity. Our preliminary data suggests this may contribute to some of the adverse complications of obese pregnancy, increased fetal size and prolonged pregnancy. A better understanding of the role of HPA axis dysregulation in adverse pregnancy outcomes may inform us which high-risk pregnancies should be targeted to improve the health of the pregnant woman and the developing baby.

3.6 Appendices

Maternal Characteristics	Obese (n=20)	Lean (n=20)	p-Value
Age (years)	32.6 (5.2)	33.5 (3.0)	0.53
BMI (kg/m ²)	43.3 (4.1)	22.4 (1.8)	<0.0001
Parity n (%)			
- Primiparous	11 (55)	10 (50)	0.88
- Para 1	7 (35)	7 (35)	
- Para ≥2	2 (10)	3 (15)	
Smoking status n (%)			
- smoker	1 (5)	0 (0)	0.40
- non-smoker	19 (95)	20 (100)	

Appendix Table 3-1. Maternal Characteristics

Parity was defined as ‘nulliparous’ (no previous pregnancies), ‘primiparous’ (no previous pregnancies delivered after 24 weeks), ‘para 1’ (one previous delivery after 24 weeks gestation) and ‘para ≥ 2’ (two or more previous deliveries after 24 weeks gestation). ‘Smokers’ were defined as those who considered themselves as current smokers. Data are mean (sd) or N(%).

Maternal Characteristics	ONP (n=7)	LNP (n=7)	OP (n=6)	LP (n=5)	P-value
Age (years)	35.2 (5.3)	25.9 (2.0)	27.1 (4.1)	35.2 (1.9)	<0.0001
BMI (kg/m ²)	40.8 (4.2)	22.6 (1.8)	42.1 (5.8)	27.1 (4.1)	<0.0001
Parity n (%)					
- Nulliparous	2	7	-	-	<0.0001
- Primiparous	2	-	2	1	
- Para 1	2	-	2	4	
- Para ≥2	-	-	2	0	
Smoking status n (%)					
- smoker	1	0	0	0	0.34
- non-smoker	6	7	6	5	

Appendix Table 3-2. Maternal characteristics

Parity was defined as ‘nulliparous’ (no previous pregnancies), ‘primiparous’ (no previous pregnancies delivered after 24 weeks), ‘para 1’ (one previous delivery after 24 weeks gestation) and ‘para ≥ 2’ (two or more previous deliveries after 24 weeks gestation). ‘Smokers’ were defined as those who considered themselves as current smokers. Data are mean (sd) or N(%).

Key: LNP (lean non-pregnant), ONP (obese non-pregnant), LP1 (lean pregnant visit 1, approximately 19 weeks gestation), LP2 (lean pregnant visit 2, approximately 36 weeks gestation), OP1 (obese pregnant visit 1, approximately 19 weeks gestation) and OP2 (obese pregnant visit 2, approximately 36 weeks gestation).

3.7 Chapter Conclusion

In support of the overarching hypothesis of this Thesis, the work presented in **Chapter 3** suggests that the HPA axis is dysregulated and in fact decreased in maternal obesity. The findings of lower maternal cortisol in obese pregnancy, without an increase in total urinary glucocorticoids suggest that the lower circulating cortisol was not due increased urinary clearance. To further support the overarching hypothesis, in obese pregnancy lower maternal cortisol tended to associate with increased birthweight and lower maternal CRH associated with longer length of gestation. Decreased HPA axis activity in maternal obesity may be a novel mechanism underlying increased birthweight and longer gestation in this high-risk population.

Chapter 3 laid the foundation for the subsequent chapters in this Thesis, which aimed to further characterise the HPA axis in obese pregnancy, and to identify potential underlying mechanisms for this dysregulation. **Chapter 3** did not examine the time of delivery. In **Chapter 4** this was investigated by studying maternal and fetal glucocorticoids from blood samples obtained at delivery.

Chapter 4

Glucocorticoids are lower at delivery in maternal, but not in cord blood of obese pregnancies

The following materials have been published in Scientific Research and Reports under the same title by Laura I Stirrat (LS), George Just (GJ), Natalie ZM Homer (NH), Ruth Andrew (RA), Jane E Norman (JN) and Rebecca M Reynolds (RR). LS designed the study, selected the samples, collated and analysed all data. GJ conducted the laboratory analysis with guidance from NH. LS prepared the first draft of the manuscript under the guidance of RR. All authors provided critical insight for the manuscript.

In summary, this work demonstrated that at the time of delivery, active glucocorticoids cortisol and corticosterone were significantly higher in maternal than cord blood. Inactive versions were significantly higher in cord than maternal blood. Increased maternal BMI associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone. Despite significant positive correlations between maternal and cord blood glucocorticoid levels, increased maternal BMI was not associated with lower cord blood glucocorticoid levels.

This work concluded that conditions at delivery may overcome any potential negative effects of low maternal glucocorticoids on the fetus in the short-term. This may not preclude the longer-term effects of fetal exposure to lower glucocorticoid levels during obese pregnancy.

4.1 Abstract

Glucocorticoids are vital for lung maturation. We previously showed that cortisol is lower in obese pregnancy. Whether this is maintained at delivery is unknown but is clinically relevant as maternal and cord blood cortisol levels are correlated and offspring of obese are more likely to need neonatal respiratory support. We hypothesized that glucocorticoids are lower in maternal and cord blood at delivery in obese pregnancies.

Glucocorticoids (cortisol and corticosterone) and their inactive versions (cortisone and 11-dehydrocorticosterone) were measured by LC-MS/MS in maternal and cord plasma from 259 Caucasian women at delivery (BMI 18-55 kg/m²). Analyses adjusted for labour status, delivery mode, offspring gender, birthweight and gestational age.

Cortisol and corticosterone were significantly higher in maternal than cord blood. Inactive versions were significantly higher in cord than maternal blood. Increased maternal BMI associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone. Despite significant positive correlations between maternal and cord blood glucocorticoid levels, increased maternal BMI was not associated with lower cord blood glucocorticoid levels.

Conditions at delivery may overcome any potential negative effects of low maternal glucocorticoids on the fetus in the short-term. This may not preclude the longer-term effects of fetal exposure to lower glucocorticoid levels during obese pregnancy.

4.2 Introduction

Glucocorticoids are vital for fetal growth and lung maturation. This has been demonstrated therapeutically when glucocorticoids are administered antenatally to women at threat of preterm labour with consequent reduced respiratory morbidity in both extremely preterm (Morgan et al., 2016), and nearer term babies (Saccone and Berghella, 2016). We and others have shown that circulating maternal cortisol levels are lower in obese compared with lean women during pregnancy (Stirrat et al., 2016, Berglund et al., 2016, Goedhart et al., 2010) and postpartum (Stirrat et al., 2016). Whether or not lower maternal cortisol levels in obese are maintained at delivery is clinically relevant as maternal and cord blood cortisol levels are correlated (Sarkar et al., 2008, Baibazarova et al., 2013, Gitau et al., 1998). As offspring born to obese women are more likely to need respiratory support at delivery (Blomberg, 2013), it is plausible that exposure to comparatively lower levels of glucocorticoids *in utero* could limit fetal lung maturation in this group. One previous study reported lower maternal cortisol, but not cord cortisol at delivery in obese compared to normal weight women (Berglund et al., 2016), but did not adjust for mode of delivery or labour; both factors are known to influence cord cortisol levels (Gitau et al., 2001, Smith et al., 2011, Vogl SE, 2006, Mears et al., 2004, Wynne-Edwards et al., 2013).

While cortisol is the major circulating glucocorticoid hormone in humans, there is increasing interest in the potential physiological roles of corticosterone, which comprises 5-10% of total plasma glucocorticoids (Nixon et al., 2016). The observation of proportionally greater increases in cord blood corticosterone than cortisol according to ‘stressful’ labour and mode of delivery has led to the suggestion that the full-term human fetus preferentially secretes corticosterone in response to fetal stress (Wynne-Edwards et al., 2013).

To our knowledge there are no studies measuring maternal corticosterone levels at time of delivery and it is not known whether corticosterone levels differ in obese and lean pregnancy. Both cortisol and corticosterone can freely cross the placenta from mother to fetus, and are metabolised to their inactive forms (cortisone and 11-dehydrocorticosterone, respectively) by the placental enzyme 11beta-hydroxysteroid dehydrogenase type 2 (11 β -HSD2).

We hypothesized that cortisol and corticosterone levels measured at the time of delivery would be lower in the maternal and cord blood of obese pregnancies than lean, even after adjusting for potentially confounding factors such as labour and mode of delivery. We tested this hypothesis by testing associations of maternal BMI with cortisol, corticosterone and their metabolites, measured in maternal and cord blood samples obtained at delivery from women across a range of obesity levels.

4.3 Method

4.3.1 Clinical Methods / Participants

We selected matched maternal and cord blood samples of 259 pregnancies from the Edinburgh Reproductive Tissue BioBank (ERTBB; ethical approval REC09/S0704/3), collected between January 2010 – December 2014. The ERTBB stores anonymised tissue specifically collected for pregnancy research with the majority of samples collected prior to the start of elective Caesarean section. Tissue samples are linked to a database containing clinical records. Our sample set comprised 102 lean (BMI 18.5 – 24.9 kg/m²), 79 overweight (BMI 25.1 – 29.9 kg/m²), 45 obese (BMI 30 – 39.0 kg/m²) and 33 severely obese (BMI \geq 40 kg/m²) women. Eligible women were Caucasian, had a singleton pregnancy, a normal booking ultrasound scan, and had not received any glucocorticoid

therapy during their pregnancy. Clinical outcomes were extracted from clinical records. Macrosomia was defined as birthweight ≥ 4000 g at term (≥ 37 weeks).

4.3.2 Biological Samples

Trained research midwives and research technicians collected maternal and umbilical cord plasma at the time of delivery. Maternal samples were obtained during the first stage of labour from women who laboured, or when intravenous access was sited prior to elective Caesarean section. Samples were collected in chilled EDTA vials and centrifuged within one hour of collection. Plasma was separated and stored at -80 °C until analysis.

4.3.3 Laboratory Methods

Plasma steroid extraction and LC-MS/MS quantification: A method was developed to measure cortisol, cortisone, corticosterone and 11-dehydrocorticosterone simultaneously by liquid chromatography tandem mass spectrometry (LC-MS/MS), using an ABSciex QTRAP® 5500 (Warrington, UK) operated in positive ion electrospray ionisation, with a Waters Acquity™ UPLC system (Manchester, UK). Analytes were extracted from plasma (200 μ L) via liquid-liquid extraction (chloroform 10:1 (v/v)) with epi-cortisol (25 ng; Steraloids, USA), epi-corticosterone (25 ng; Steraloids, USA) and 9,11,12,12 [$^2\text{H}_4$] cortisol (d_4 -cortisol; 25 ng; QMX Laboratories, UK) included as internal standards. Analytes were separated at 40 °C on a Waters Sunfire™ C18 (2.1 x 150 mm; 3.5 mm) column (Manchester, UK) using an isocratic solvent system (70:30 of water with 0.1% formic acid and acetonitrile with 0.1% formic acid) with a gradient run of 7.1 minutes. Mass spectral conditions are demonstrated in **Appendix Tables 4-1 and 4-2**. Inter-assay precision and accuracy were within

acceptable limits (**Appendix Table 4-3**). The ratio of cortisol: cortisone was examined as a marker of 11 β -HSD2 enzyme activity.

4.3.4 Statistical Analysis

Data distribution was assessed for normality by visually assessing histograms. Data that were not normally distributed were normalized using the natural-log transformation. The independent t-test was used to test for differences between continuous variables and chi-squared test for categorical variables. The one-way ANOVA was used to compare change in hormone levels between different groups. Simple linear regression was used to test for associations between maternal and cord blood glucocorticoid levels, and maternal BMI as a continuous variable (unadjusted beta; β). Using multiple linear regression analysis we adjusted (β_a) for covariates and confounding factors known to influence glucocorticoid levels including mode of delivery or labour (McTernan et al., 2001, Murphy and Clifton, 2003), and gestational age (Sandman et al., 2006, Nwosu et al., 1975), or that differed between groups in our sample such as offspring birthweight. Model 1 adjusted for gestational age at delivery, offspring birthweight and mode of delivery. Model 2 adjusted for gestational age at delivery, offspring birthweight and labour status (labour or non-labour). Analysis was performed using SPSS v21 (IBM). Data in text are mean \pm sd, and data in figures are mean \pm SEM. Statistical significance was considered at $p < 0.05$.

4.4 Results

4.4.1 Demographics

Maternal and neonatal characteristics are demonstrated in **Table 4-1**. Obese and severely obese women were younger than lean women. Severely obese women had the highest numbers of current smokers. Women were well matched for parity, mode of delivery, gestational age at delivery and offspring gender. The most common mode of delivery in all BMI groups was elective Caesarean section, representative of samples in the ERTBB. Induction of labour was highest in the severely obese group. Offspring birthweight was highest in the obese and severely obese groups. Lean women had significantly lower birthweight than both obese and severely obese women offspring. Rates of macrosomia were highest in the obese and severely obese group.

	Lean (n=102)	Overweight (n=79)	Obese (n=45)	Severely Obese (n=33)	p-value
Maternal age (years)	34.2 (5.0)	34.6 (6.2)	32.0 (5.2)	31.7 (5.0)	0.017
Maternal BMI (kg/m ²)	22.6 (1.6)	27.3 (1.4)	34.2 (2.8)	44.3 (3.6)	<0.0001
Parity n (%)					
Prim	27 (26.5)	13 (16.5)	8 (18.8)	8 (24.2)	0.15
Para 1	50 (49.0)	48 (60.8)	26 (57.8)	12 (36.4)	
Para ≥2	25 (24.5)	18 (22.7)	11 (24.4)	13 (39.4)	
Smoking n (%)					
Non smoker	96 (94.1)	75 (94.9)	44 (97.8)	22 (66.7)	<0.0001
Smoker	6 (5.9)	4 (5.1)	1 (2.2)	11 (33.3)	
Labour onset n (%)					
Spontaneous	3 (2.9)	3 (3.8)	3 (6.7)	2 (6.1)	0.019
Induced	2 (2.0)	0 (0)	1 (2.2)	4 (12.1)	
Prelabour CS	97 (95.1)	76 (96.2)	41 (91.1)	27 (81.8)	
Labour Type n (%)					
Labour	9 (8.8)	3 (3.8)	3 (6.7)	5 (15.2)	0.212
Non-labour	93 (91.2)	76 (96.2)	42 (93.3)	28 (84.8)	
Mode of Delivery n (%)					
ElCS	93 (91.2)	76 (96.2)	42 (93.3)	28 (84.8)	0.102
SVD	0	0	0	1 (3.0)	
Instrumental	2 (2.0)	0	1 (2.2)	2 (6.0)	
EmCS in labour	3 (2.9)	3 (3.8)	2 (4.4)	2 (6.0)	
Prelabour EmCS	4 (3.9)	0	0	0	
Gestational age at delivery (days)					
Mean (sd)	273.7 (8.1)	275.3 (7.0)	275.4 (8.5)	276.3 (7.6)	0.285
Baby gender n (%)					
Male	55 (53.4)	40 (50.6)	27 (60)	20 (60.1)	0.607
Female	47 (46.1)	39 (49.4)	18 (40)	12 (36.4)	
Birthweight					
Mean (sd)	3442 (492)	3514 (546)	3851 (642)	3739 (555)	<0.0001
Macrosomia n (%)					
Yes	12 (11.8%)	11 (13.9)	20 (44.4%)	9 (26.5%)	<0.0001

Table 4-1. Maternal and Neonatal Characteristics

Parity was defined as ‘prim’ (no previous pregnancies delivered after 24 weeks), ‘para 1’ (one previous delivery after 24 weeks gestation) and ‘para ≥ 2’ (two or more previous deliveries after 24 weeks gestation). ‘Smokers’ were defined as those who considered themselves as current smokers. Macrosomia was defined

as ≥ 4000 g. Data are mean (sd) or N (%). P-value is from one-way ANOVA for continuous variables, and chi-squared test for categorical variables.

Missing data: n=1 baby gender missing from offspring of severely obese

Key: BMI (body mass index), CS (Caesarean section), ElCS (elective Caesarean section), EmCS (Emergency Caesarean section), SVD (spontaneous vaginal delivery).

4.4.2 Relationship Between Maternal and Cord Hormone Levels

Levels of cortisol and corticosterone were significantly higher in maternal blood than cord blood, whereas cortisone and 11-dehydrocorticosterone were significantly higher in cord blood than maternal blood (**Figure 4-1**).

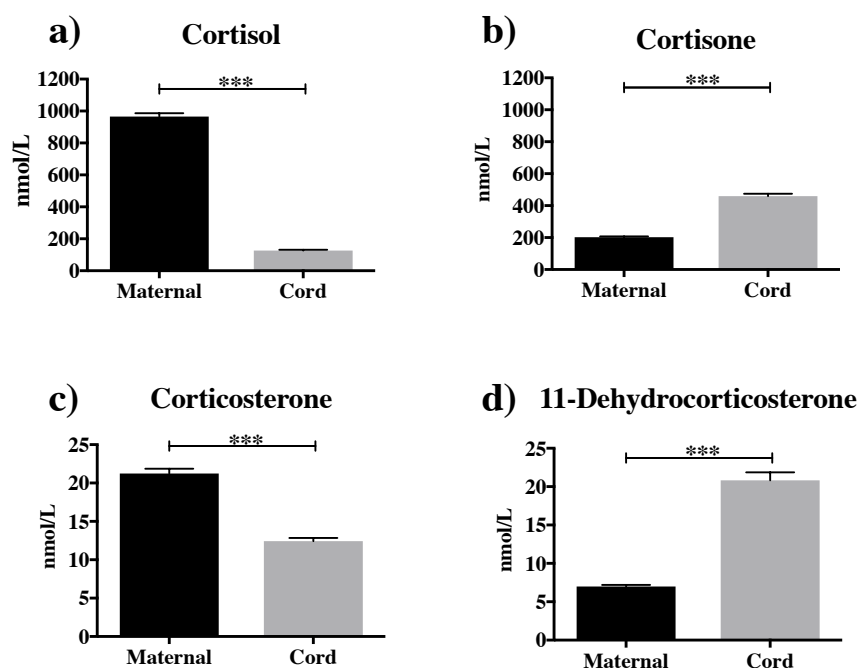


Figure 4-1 (a-d). Maternal and cord plasma levels of glucocorticoids

Cortisol (1a), cortisone (1b), corticosterone (1c) and 11-dehydrocorticosterone (1d). Data are mean (sem); ***p<0.0001.

Maternal and cord blood levels of all the monitored steroid hormones were significantly positively correlated (**Figure 4-2**). Maternal cortisol and corticosterone were also positively correlated with cord blood cortisone and 11-dehydrocorticosterone levels respectively ($r=0.454$, $p<0.001$; $r=0.437$, $p<0.001$).

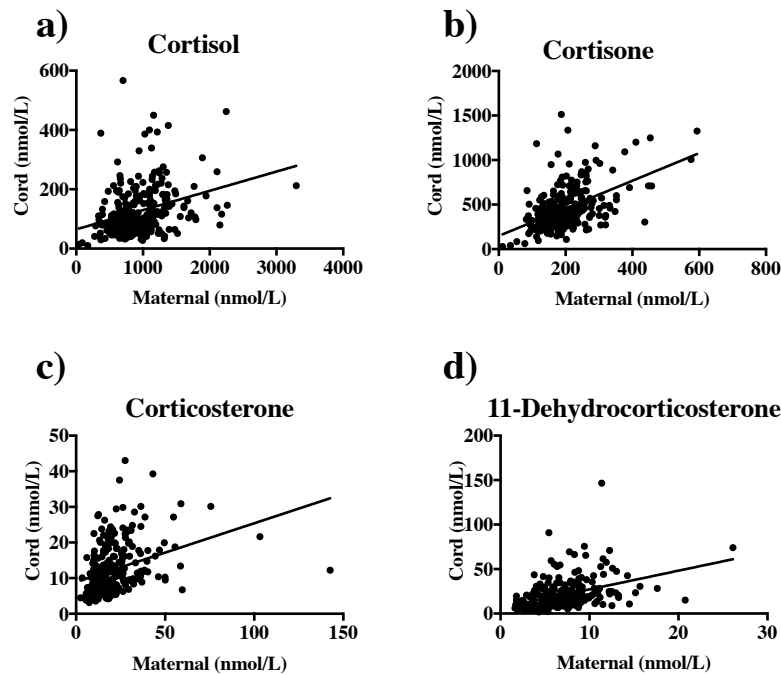


Figure 4-2 (a-d). Correlations of maternal and cord blood levels of glucocorticoids

Cortisol ($r=0.392$, $p<0.0001$; 1a), cortisone ($r=0.432$, $p<0.0001$; 1b), corticosterone ($r=0.437$, $p<0.0001$; 1c), 11-dehydrocorticosterone ($r=0.481$, $p<0.0001$; 1d).

4.4.3 Predictors of Glucocorticoid Levels at Delivery

None of the hormones differed according to parity, maternal smoking or maternal age. Maternal corticosterone was significantly higher in labouring than non-labouring deliveries and was associated with greater ‘stress’ (vaginal delivery higher than emergency and elective Caesarean sections) (**Table 4-2**). Cord corticosterone was significantly higher in labouring than non-labouring deliveries but did not differ with mode of delivery. Cord cortisone was

significantly higher in labouring than non-labouring, and was significantly higher in deliveries associated with greater 'stress' (vaginal delivery higher than elective Caesarean section).

The association of maternal BMI with glucocorticoid hormone levels is demonstrated in **Table 4-3**. In the unadjusted analysis, increased maternal BMI was associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone. Maternal BMI remained significant as an independent predictor of these hormones in the adjusted analyses (**Table 4-3**). In cord blood, there were no associations between maternal BMI and glucocorticoid levels in the unadjusted or adjusted analyses. **Table 4-4** demonstrates maternal and cord blood glucocorticoids levels in women who were delivered by elective Caesarean section, in different BMI groups. Maternal glucocorticoid levels tended to decrease with increasing maternal BMI.

Blood Type	Hormone	Labour Status			Mode of Delivery			
		Labour (n=16)	Non-labour (n=243)	p-value	Elective CS (n=239)	Emergency CS (n=14)	Vaginal delivery (n=6)	p-value
Maternal Blood	11-dehydrocorticosterone	7.7 (3.8)	6.9 (3.1)	0.59	6.9 (3.1)	6.8 (3.4)	9.9 (4.2)	0.099
	Corticosterone	34.1 (30.2)	20.1 (11.6)	0.006	20.1 (11.6)	24.0 (16.3)	57.4 (43.0)	<0.0001
	Cortisone	198.7 (77.8)	202.5 (77.4)	0.857	202.1 (77.4)	184.9 (57.9)	230.9 (111.7)	0.637
	Cortisol	1086.3 (558.4)	928.7 (398.0)	0.293	928.7 (398.0)	1026.6 (597.2)	1225.7 (473.5)	0.266
Cord Blood	11-dehydrocorticosterone	26.3 (19.7)	20.4 (16.5)	0.076	20.4 (16.5)	22.4 (15.1)	35.5 (27.3)	0.072
	Corticosterone	15.5 (7.6)	12.2 (6.8)	0.046	12.2 (6.8)	14.2 (7.4)	18.4 (8.0)	0.058
	Cortisone	661.2 (342.5)	442.8 (226.6)	0.002	442.8 (226.6)	612.7 (332.9)	774.4 (368.3)	0.004
	Cortisol	145.7 (100.1)	124.4 (83.0)	0.35	124.4 (83.0)	140.1 (114.9)	158.9 (58.6)	0.364

Table 4-2 Hormone levels by labour status and mode of delivery

Data are mean (sd). P-value from Student's t-test for labour status, and from One-way ANOVA for mode of delivery.

Blood Type	Steroid Hormone	Unadjusted		Model 1		Model 2	
		β 95% CI	P	β_a 95% CI	P _a	β_a 95% CI	P _a
Maternal Blood	11-DHC	-0.011 (-0.019 to -0.004)	0.003	-0.014 (-0.022 to -0.007)	<0.0001	-0.014 (-0.022 to -0.007)	<0.0001
	Corticosterone	-0.010 (-0.019 to -0.002)	0.019	-0.12 (-0.021 to -0.004)	0.006	-0.013 (-0.022 to -0.004)	0.004
	Cortisone	0.0000052 (-0.007 to 0.007)	0.999	-0.003 (-0.009 to 0.004)	0.442	-0.003 (-0.009 to 0.004)	0.448
	Cortisol	-0.009 (-0.017 to -0.001)	0.023	-0.011 (-0.019 to -0.003)	0.009	-0.011 (-0.019 to -0.003)	0.007
Cord Blood	11-DHC	0.004 (-0.007 to 0.016)	0.472	0.005 (-0.007 to 0.016)	0.711	0.004 (-0.008 to 0.016)	0.358
	Corticosterone	0.000058 (-0.008 to -0.009)	0.989	-0.001 (-0.010 to 0.007)	0.739	-0.002 (-0.011 to 0.007)	0.672
	Cortisone	0.006 (-0.003 to 0.016)	0.168	0.005 (-0.004 to 0.014)	0.306	0.004 (-0.005 to 0.014)	0.377
	Cortisol	0.005 (-0.005 to 0.016)	0.303	0.002 (-0.009 to 0.012)	0.769	0.001 (-0.009 to 0.012)	0.800

Table 4-3 Associations of maternal BMI with hormone levels.

Model 1: adjusted for gestational age at delivery, offspring birthweight and mode of delivery. Model 2: adjusted for gestational age at delivery, offspring birthweight and labour status (labour or non-labour).

Key: 11-DHC (11-dehydrocorticosterone)

Blood Type	Steroid Hormone	Lean (n=93)	Overweight (n=76)	Obese (n=42)	Severely Obese (n=29)	p-value
Maternal Blood	11-DHC	7.0 (3.6)	7.3 (2.9)	6.9 (2.4)	5.8 (2.8)	0.09
	Corticosterone	21.5 (14.3)	21.0 (10.8)	16.9 (6.2)	17.9 (9.0)	0.25
	Cortisone	201.7 (85.0)	205.2 (73.8)	189.9 (50.0)	216.9 (93.6)	0.81
	Cortisol	964.4 (414.6)	971.6 (472.2)	861.8 (309.5)	794.4 (351.0)	0.30
Cord Blood	11-DHC	20.2 (20.2)	20.4 (14.6)	21.2 (15.1)	19.4 (9.3)	0.65
	Corticosterone	11.8 (6.9)	12.2 (6.9)	12.5 (6.9)	13.0 (6.9)	0.89
	Cortisone	425.3 (234.3)	451.0 (229.2)	423.5 (165.4)	505.1 (239.7)	0.37
	Cortisol	116.7 (88.0)	126.1 (69.2)	143.3 (94.3)	116.9 (82.9)	0.25

Table 4-4 Maternal and cord blood hormone levels in women delivered by Elective Caesarean Section

Data are mean (sd). One-way ANOVA of natural-logged values.

4.5 Discussion

Our findings demonstrate that increased maternal BMI was associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone measured at time of delivery. Despite significant positive correlations between maternal and cord blood hormone levels, maternal BMI was not associated with cord blood glucocorticoid levels.

Our observation that cortisol and corticosterone were higher in maternal than cord blood, and that cortisone and 11-dehydrocorticosterone were higher in cord than maternal blood supports the hypothesis that the placenta acts as a barrier to protect the fetus from overexposure to active glucocorticoids (Benediktsson et al., 1997). Indeed, maternal cortisol and corticosterone were positively correlated with cortisone and 11-dehydrocorticosterone respectively. We also found a significant positive correlation between maternal and cord hormone levels (for all hormones), consistent with studies showing that maternal plasma and amniotic fluid cortisol levels are correlated during pregnancy (Sarkar et al., 2008, Baibazarova et al., 2013, Gitau et al., 1998, Glover et al., 2009).

Our findings are consistent with literature showing that labour and mode of delivery are associated with both a maternal and fetal endocrine (glucocorticoid hormone) stress response. For example, both higher maternal and cord blood cortisol levels have been reported where delivery was by vaginal or emergency Caesarean section (i.e. where labour had occurred), compared with delivery by elective Caesarean section (i.e. pre-labour) (Vogl SE, 2006, Wynne-Edwards et al., 2013, Gitau et al., 2001). While it is generally considered that higher glucocorticoid levels are a consequence of 'stressful' conditions at delivery such as labour, an alternative hypothesis could be that higher glucocorticoid levels could be linked to the 'cause' of labour. Glucocorticoids are thought to play a role in human parturition (Li et al., 2014), although the mechanisms are not known.

Our finding of higher corticosterone (but not cortisol) in labouring cord samples was in-keeping with a previous study (Wynne-Edwards et al., 2013) who found highest corticosterone levels in vaginal deliveries, followed by emergency Caesarean deliveries (who would have laboured), and lowest levels in elective Caesarean

deliveries (no labour). It has been suggested that this may be due to developmental changes in fetal glucocorticoid synthesis with preferential secretion of corticosterone in utero, and a shift towards the adult pattern of preferential cortisol synthesis after delivery (Wynne-Edwards et al., 2013). These observations suggest that fetal adrenal corticosterone may be a better marker of fetal stress (Wynne-Edwards et al., 2013). In addition, we showed for the first time that maternal corticosterone levels were higher in labouring than non-labouring women, which may suggest this hormone is released into the maternal circulation in response to the 'stress' associated with labour. However, we did not replicate previous findings of higher cord cortisol in cases where labour has occurred (Gitau et al., 2001, Smith et al., 2011, Vogl SE, 2006); a large proportion of our subjects were delivered by elective Caesarean section, and more labouring samples may be required to replicate this finding.

Smith et al., (Smith et al., 2011) found no effect of maternal mood or anxiety disorders on cord blood cortisol at delivery, and suggested that conditions at delivery *per se* overwhelm the possible smaller diagnosis of treatment-related differences in hypothalamic-pituitary-adrenal axis responses during pregnancy. However, they did not adjust for mode of delivery or labour. Our data suggest that even in the 'controlled' conditions of elective Caesarean section, in accord with these findings (Smith et al., 2011) the conditions at delivery *per se* may overwhelm the effects of BMI on cord cortisol. While detailed studies conducted at elective Caesarean section have informed our knowledge of maternal, placental and fetal glucose transfer (Holme et al., 2015) such studies are less likely to be informative about glucocorticoid transfer between the maternal, placental and fetal unit.

In rodents, corticosterone is the major circulating glucocorticoid hormone and a handful of studies have measured maternal and fetal glucocorticoid levels in animal models of obesity in pregnancy. Two studies in mice and rats fed a high-fat diet reported increased levels of maternal corticosterone (Bellisario et al., 2015, Desai et al., 2014) suggesting that a high-fat diet acts as a stressful challenge during rodent pregnancy. However, in contrast to both of these studies, another study in rats measured rhythmic 21-hour profiles of maternal and fetal corticosterone and 11-dehydrocorticosterone, and found that these were unaffected by obesity (Crew et al., 2016). Whilst rodent studies can more easily control for complications of labour and

delivery than human studies and so are important comparisons for the effect of obesity on glucocorticoid levels, the species in maternal glucocorticoid responses to obesity/high fat diet need to be considered in interpreting the observations.

Strengths of our study are the large sample size, with a high proportion of elective Caesarean deliveries, and a wide range of maternal BMI. Our finding of higher birthweight in offspring of obese women is representative of what is expected in this sub-population of pregnant women (Ehrenberg et al., 2004). Our samples were analysed by liquid chromatography tandem mass spectrometry, which is the gold standard for measuring glucocorticoid hormones, and facilitated the simultaneous analysis of multiple hormones from a small sample volume (200 μ L). We are also the first to describe the relationship of all these glucocorticoid hormones between maternal and cord blood.

A limitation of this study is that, like others (Vogl SE, 2006, Smith et al., 2011) our cord blood samples were mixed cord artery and vein meaning we were unable to conduct a detailed assessment of the placental and fetal contribution to corticosterone metabolism. This was due to limited availability of cord blood in our setting, where delayed cord clamping is practiced and clinical bloods are required for blood gas testing; thus, the volume of blood available for research purposes was limited. Some studies have demonstrated differences in levels of cortisol and corticosterone between cord artery and vein (Gitau et al., 2001, Wynne-Edwards et al., 2013). For example, in a study of 256 matched arterial and venous cord blood samples, Wynne-Edwards et al., reported an increase in both cortisol and corticosterone from the venous to the arterial circulation, suggesting that the fetal adrenal contributed corticosteroids to the arterial circulation while placental 11 β -HSD2 was clearing both corticosteroids (Wynne-Edwards et al., 2013). *Ex vivo* studies using the placental perfusion model (Benediktsson et al., 1997) combined with deuterated cortisol tracers (Andrew et al., 2002) would allow more details assessment of the fetal and placental contribution to corticosteroid levels.

In conclusion, our study suggests that maternal glucocorticoids are lower at delivery in obese pregnancy, and that conditions at delivery may overwhelm any effect of BMI on cord glucocorticoid levels. This should be considered in studies investigating glucocorticoids at delivery. Though conditions at delivery may overcome any

potential negative effects of low glucocorticoids on the fetus in the short-term, this may not preclude the longer-term effects of lower glucocorticoid exposure during obese pregnancy and further studies are needed to examine this.

4.6 Appendices

	Molecular Weight (Da)	Precursor ion (<i>m/z</i>)	Product ion Quan; Qual	Declustering potential (V)	Collision energy (V) Quan; Qual	Cell exit potential (V) Quan; Qual
ANALYTES						
Cortisol	362.5	363.2	121, 77	131	29, 101	14, 14
Cortisone	360.4	361.2	77; 163.2	166	99; 11	36; 20
Corticosterone	346.5	347.1	121.1; 97.1	66	69; 69	8; 8
11-dehydrocorticosterone	344.4	345.1	121.2; 90.9	51	33; 71	8; 10
INTERNAL STANDARDS						
D ₄ -cortisol	366.5	367.0	121; only one	121	25	20
Epi-cortisol	362.5	363.2	121; only one	131	29	14
Epi-corticosterone	346.5	347.1	121; only one	66	69	8

Appendix Table 4-1. Mass spectral conditions for analysis of steroid hormones and internal standards using positive ion electrospray ionisation

Key: Da (Daltons), Quan (quantifier ion), Qual (qualifier ion), V (volts)

	Standard curve range (nmol/L)	LLOQ (nmol/L)
Cortisol	1.4 - 2762	3.5
Cortisone	1.4 - 2906	6.9
Corticosterone	1.5 - 2777	3.6
11-Dehydrocorticosterone	1.4 - 2890	3.6

Appendix Table 4-2. Lower limits of quantitation

Key: LLOQ (lower limits of quantitation)

	Target Concentration (nmol/L)	Inter-assay (n=6)		
		Concentration (nmol/L): mean (SD)	Precision (% RSD)	Accuracy (%)
Cortisol	Low (3.5)	3.7 (0.6)	14.7	108
	Mid (691)	742 (35)	2.5	107
	High (2762)	2463 (177)	7.2	89
Cortisone	Low (3.5)	7.2 (1.4)	18.7	105
	Mid (694)	718 (43)	6.0	103
	High (2778)	2467 (171)	6.9	89
Corticosterone	Low (3.6)	4.3 (0.7)	15.5	99
	Mid (723)	725 (46.2)	6.3	100
	High (2980)	2754 (98.3)	3.6	95
11-dehydrocorticosterone	Low (3.6)	3.8 (0.4)	13.0	105
	Mid (727)	794 (76)	3.4	104
	High (2906)	2602 (291)	11.2	89

Appendix Table 4-3. Inter-assay precision and accuracy

4.7 Chapter Conclusion

The work in **Chapter 4** suggests that the finding of lower maternal glucocorticoids during the antenatal period of obese pregnancy in **Chapter 3**, is maintained in maternal blood at the time of delivery. Increased maternal BMI associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone. However, despite significant positive correlations between glucocorticoids levels in maternal and cord blood, increased maternal BMI did not associate with lower cord blood glucocorticoid levels. It was suggested that in the short-term, conditions at delivery may overcome potential negative effects of lower maternal glucocorticoids on the fetus.

Potential underlying mechanisms for the pattern of lower maternal glucocorticoids and decreased HPA axis activity described in **Chapters 3 and 4**, were investigated and described in **Chapters 5 and 6**. **Chapter 5** relates to a clinical study that examined the effects of maternal obesity on the release of glucocorticoids.

Chapter 5

Pulsatility of Glucocorticoids Hormones in Pregnancy: Changes with Gestation and Obesity

The following materials are in revision in Clinical Endocrinology under a similar title by Laura I Stirrat (LS), Jamie J Walker (JW), Ksenia Stryjakowska (KS), Natalie Jones (NJ), Natalie ZM Homer (NH), Ruth Andrew (RA), Jane E Norman (JN), Stafford L Lightman (SL) and Rebecca M Reynolds (RR). LS designed the study, applied for ethical approval, recruited participants, conducted study visits, laboratory analysis, collated and analysed data; with guidance from RR and JN. JW conducted the analysis of pulsatility using the Cluster algorithm. KS assisted with recruitment and study visits for six months. NJ optimised the liquid chromatography tandem mass spectrometry assay with guidance of NH and RA. LS prepared the first draft of the manuscript under the guidance of RR. All authors provided critical insight for the manuscript.

In summary, this work demonstrated that serum cortisol AUC (measured during six day-time hours) increased significantly with gestation in lean and obese pregnant compared with non-pregnant subjects. Peripheral tissue interstitial fluid cortisol was found to be pulsatile in human pregnancy. Pulse frequency was decreased with increasing gestation in obese pregnant women. These findings may be potential mechanisms underlying decreased HPA axis activity in obese pregnancy.

5.1 Abstract

Background: Hypothalamic-pituitary-adrenal-axis (HPA) activity is decreased in obese pregnancy and associates with increased fetal size. Pulsatile release of glucocorticoid hormones regulates their action in target tissues. Glucocorticoids are essential for normal fetal growth but little is known about glucocorticoid pulsatility in pregnancy. We aimed to investigate the ultradian rhythm of glucocorticoid secretion during obese and lean pregnancy and non-pregnancy.

Method: Serum cortisol, cortisone, corticosterone and 11-dehydrocorticosterone were measured by LC-MS/MS from samples obtained at 10-minute intervals between 08.00-11.00h and 16.00h-19.00h, from eight lean ($\text{BMI} < 25 \text{ kg/m}^2$) and seven obese ($\text{BMI} > 35 \text{ kg/m}^2$) pregnant women between 16-24 weeks gestation and again at 30-36 weeks), and non-pregnant controls (lean $n=3$, obese $n=4$) during the luteal phase of their menstrual cycle. Interstitial fluid cortisol was measured by ELISA, from samples obtained using a portable microdialysis and automated collection device at 20-minute intervals over 24-hours.

Results: Serum cortisol AUC, highest peak, and lowest trough increased significantly with gestation in lean and obese pregnant compared with non-pregnant subjects. Pulsatility of cortisol was detected in interstitial fluid. In pregnant subjects, interstitial fluid pulse frequency was significantly lower with advancing gestation in obese, but not in lean.

Conclusions: We demonstrate cortisol pulsatility in interstitial fluid. Pulse frequency is altered with increased gestation and BMI. This may be a novel mechanism to explain decreased HPA activity in obese pregnancy.

Keywords: Cortisol, pulsatility, pregnancy, obesity

5.2 Introduction

Glucocorticoids are vital for normal fetal growth and organ development, but fetal over-exposure to the major circulating glucocorticoid, cortisol, is associated with intrauterine growth restriction (Stewart et al., 1995), and an increased risk of cardiovascular disease in later life (Reynolds, 2013). During pregnancy a number of endocrine changes cause the maternal hypothalamic-pituitary-adrenal (HPA) axis to undergo dramatic activation resulting in cortisol levels that are around three-fold higher than in non-pregnancy (Duthie and Reynolds, 2013, Jung et al., 2011, Lindsay and Nieman, 2005). Dysregulation of the maternal HPA axis has been implicated in pregnancy complications including preterm birth (Smith, 2007) and pre-eclampsia (Aufdenblatten et al., 2009, Vianna et al., 2011). The glucocorticoid corticosterone, which is known to circulate at significantly lower levels than cortisol in non-pregnancy (Raubenheimer et al., 2006) is increasingly being recognised as a key player in HPA axis regulation (Nixon et al., 2016). It has been suggested that the fetus preferentially secretes corticosterone over cortisol in response to stress, and therefore has been proposed to be an important biomarker of fetal stress at the time of delivery (Wynne-Edwards et al., 2013, Stirrat et al., 2017), but little is known about how it changes during pregnancy.

The circadian rhythm of the HPA axis, which in humans is characterised by peak levels of cortisol early in the morning, is maintained in pregnancy (de Weerth and Buitelaar, 2005). More detailed studies of circadian rhythmicity in animals (Droste et al., 2008) and in non-pregnant humans (Bhake et al., 2013) have identified an underlying ‘ultradian rhythm’ (Veldhuis et al., 1989) of cortisol pulses within blood and target tissues (Spiga et al., 2014), occurring approximately once per hour (Veldhuis et al., 1989, Follenius et al., 1987, Jasper and Engeland, 1991). Pulse amplitude and frequency increase during the circadian peak of secretion when circulating cortisol levels are at their highest (Veldhuis et al., 1989), and in response to food (Fall et al., 2002). These pulses are important for optimal gene transcription and metabolic functions (Lightman and Conway-Campbell, 2010). Altered ultradian rhythm patterns have been linked to pathological consequences and manifestations of disease (Lightman and Conway-Campbell, 2010) such as psychotic and depressive states (Young et al., 1994, Young et al., 2004), where elevated troughs at the nadir

result in a flattened circadian rhythmicity. In pregnancy, excess glucocorticoid exposure is thought to induce a long lasting effect on peripheral tissue expression of glucocorticoid sensitive genes (Cottrell and Seckl, 2009). However, the underlying mechanisms for such changes are unknown. One study of cortisol pulsatility in pregnancy used 30-minute serum sampling and detected 2-3 pulses of cortisol in 12 hours in the third trimester of human pregnancy (Magiakou et al., 1996), but the authors acknowledged that 30-minute sampling was suboptimal for accurate detection of pulse rates of hormones with rapid clearance rates. Whether or not and how the ultradian rhythm of cortisol secretion changes across gestation is unknown. This information will improve understanding of pathways to fetal growth as well as the aetiology of pregnancy complications.

We aimed to determine whether there are changes in cortisol pulsatility in two physiological contexts of altered HPA axis activity; firstly the increase in circulating cortisol levels that occur with advancing gestation, and secondly in maternal obesity, where we and others have reported lower maternal circulating cortisol levels in obese than in lean pregnant women (Stirrat et al., 2016, Berglund et al., 2016). We hypothesised that pulse amplitude and frequency would increase with advancing gestation, and that these characteristics would be lower in obese compared with lean pregnant women. To test this hypothesis we studied day-time serum profiles of both cortisol and corticosterone as well as their inactive metabolites (cortisone and 11-dehydrocorticosterone, respectively), and 24-hour interstitial fluid cortisol levels in obese and lean pregnant, and non-pregnant women. We conducted more frequent sampling than has previously been performed (Magiakou et al., 1996) to maximise the likelihood of detecting cortisol pulses.

5.3 Method

5.3.1 Participants and Clinical Protocol

We recruited lean (BMI 18.5-24.9 kg/m²) pregnant (LP; n=8), obese (BMI \geq 30 kg/m²) pregnant (OP; n=7), lean non-pregnant (LNP; n=3) and obese non-pregnant

(ONP; n=4) women. One obese participant was studied in non-pregnancy and again during pregnancy. Pregnant women were recruited from antenatal clinics in NHS Lothian. Non-pregnant volunteers were recruited from the University of Edinburgh, and community weight loss clinics in NHS Lothian. Eligible pregnant women were Caucasian, had a normal booking ultrasound scan and a singleton pregnancy. Non-pregnant women had a regular menstrual cycle, and did not use hormonal contraception. Exclusion criteria were smoking, pre-existing diabetes, regular glucocorticoid medication, severe mental health disorder, and anaemia. Ethical approval and written informed consent were obtained.

Participants attended study visits at the Edinburgh Clinical Research Facility, Royal Infirmary of Edinburgh. Pregnant women attended for study visits at 16-24 weeks' and between 30-36 weeks' gestation, and non-pregnant volunteers attended for one study visit in the luteal phase of the menstrual cycle.

At each study visit, fasting blood samples were obtained at 10-minute intervals between 08.00h-11.00h and between 16.00h-19.00h via a peripheral venous cannula. Participants were fasted from bed-time the night before each study visit, then were fed a standardized breakfast meal after completion of sampling at 11.00h (toast, cup of tea or coffee, and carton of fruit juice), and lunch at 13.00h (sandwich, yoghurt, piece of fruit, cup of tea or coffee and biscuit). Fasting was re-commenced by 15.00h until after completion of the second sampling session at 19.00h. Participants could drink water freely throughout study visits.

Subcutaneous interstitial fluid samples for measurement of free cortisol (Sandeep et al., 2005) were obtained at 20-minute intervals over 24 hours by microdialysis. A linear microdialysis catheter (Linton, Norfolk, UK) was inserted subcutaneously into the interstitial compartment of the anterior abdominal wall, and collected via a novel, miniaturised, portable collection device as described by Bhake et al., 2013 (Bhake et al., 2013).

Serum was separated immediately and stored at -80 °C for later laboratory analysis. Clinical outcomes including maternal booking BMI, gestation at delivery, birthweight and offspring gender were extracted from medical records.

5.3.2 Laboratory Methods

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS): We measured cortisol, cortisone, corticosterone and 11-dehydrocorticosterone simultaneously by liquid chromatography tandem mass spectrometry, using a Waters Acquity™ UPLC (Manchester, UK), liquid chromatography system followed by mass spectral analysis on an ABSciex QTRAP® 5500 (Warrington, UK) mass spectrometer. Mass spectral conditions are described in **Appendix Table 5-1** in conjunction with ion spray voltage (5500 V) and source temperature (700 °C). Following enrichment of serum (200 mL) with internal standards epi-cortisol (25 ng; Steraloids, USA), epi-corticosterone (25 ng; Steraloids, USA), and 9,12,12,12 [²H₄]-cortisol (D4-cortisol 25 ng; QMX Laboratories, England, UK), and dilution with water (200 mL) analytes were extracted via supported liquid extraction (ISOLUTE® SLE+ 400 µl 96-well plate, Biotage, Sweden) and eluted with 98:2 dichloromethane:2-propanol. Analytes were separated on an ACE Excel 2 C18-AR (150 x 2.1 mm, 2 µm) column (Advanced Chromatography Technologies Ltd, UK) at 40°C. The elution process started with 70:30 water with 0.1% formic acid (FA) (solution A) and acetonitrile with 0.1% FA (solution B), was maintained for 4 minutes followed by a 1-minute linear rise to 60% solution B, a subsequent rise to 90% solution B, before returning to 30% solution B by 6.1 minutes at a constant flow rate of 0.5 mL/min.

Validation parameters (intra- and inter-assay precision, accuracy; **Appendix Table 5-2**) were within acceptable limits (the lowest levels being acceptable < 20% RSD with all following < 15%). Levels of corticosterone and 11-dehydrocorticosterone in some samples were close to or below the limit of quantification in all subjects, and at all gestations of pregnancy. The ratios of cortisol: cortisone and corticosterone: 11-dehydrocorticosterone were used as a marker of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme activity.

Commercially available ELISA kits were used for the analysis of plasma ACTH (Demeditec DE3467; inter-assay CV 6.9-7.1%), and interstitial fluid cortisol (IBL International RE52611; inter-assay CV 4.2-17.0%), as previously used by Bhake et al. (Bhake et al., 2013) Free cortisol values were expected to be above the top standard, therefore were diluted to 1:10 in the assay zero standard and results were corrected for dilution.

5.3.3 Statistical Analysis

Data distribution was assessed for normality by visually assessing histograms. Data that were not normally distributed were normalised using the natural-log transformation. The independent t-test was used to test for differences in subject characteristics between continuous variables and chi-squared test for categorical variables. For cortisol measurements the 'highest peak' was the highest value in any given profile, and the 'lowest trough' was the lowest value. The area under the curve (AUC) for hormone profiles was used as a marker for total glucocorticoid levels over the study period. Pulse analysis was performed using Cluster analysis (Veldhuis and Johnson, 1986), a statistically-rigorous peak detection algorithm that has been widely used to quantify the pulsatile dynamics of various hormones, including cortisol (van Aken et al., 2005). Cluster analysis defines a pulse as a statistically significant increase in a 'cluster' of hormone values followed by a statistically significant decrease in a second cluster of values. The increase and decrease are judged in relation to the actual experimental error expressed by replicates in the presumptive nadir and peak results. The algorithm detected statistically-significant interstitial fluid cortisol pulses, pulse frequency (pulses/hr), pulse height (ug/dL) and mean concentration (ug/dL) in a given cortisol time series. Cluster parameters used in the analysis were as follows: minimum detectable concentration of the assay (MDC, 0.015 ug/dL); intra-assay coefficient of variation (CV, 12.08%); test cluster size for sliding nadir (2.0); test cluster size for sliding peak (1); t-statistic for significant increase in the data (2.0); t-statistic for significant decrease in the data (2.0); minimum peak size (0.0 ug/dL). One-way ANOVA was used to compare changes in pulse characteristics between different groups. The paired t-test was used to assess differences in pulse characteristics between pregnant women who attended for two study visits. Analysis was performed using SPSS v21 (IBM). Data in text are mean \pm (sd), and data in figures are mean \pm SEM. Statistical significance was considered at $p < 0.05$.

5.4 Results

5.4.1 Demographics

Maternal and neonatal characteristics are demonstrated in **Table 5-1**. In pregnant participants, OP were younger and had higher systolic and diastolic blood pressure than LP. The timing of study visit 1 tended to be earlier in LP, and gestation at delivery was earlier in OP. There were no differences in parity, birthweight percentile or standard deviation birthweight score. In non-pregnant participants, obese subjects were older, and also had higher systolic and diastolic blood pressure than lean.

		Non-pregnant			Pregnant		
		Lean (n=3)	Obese (n=4)	p-value	Lean (n=8)	Obese (n=7)	p-value
Demographics	Age (years)	36.3 (4.2)	39.0 (4.1)	0.435	35.1 (1.7)	29.3 (5.3)	0.026
	BMI (kg/m ²)	22.3 (2.1)	36.5 (3.3)	0.001	21.9 (1.6)	43.7 (5.3)	<0.0001
	Weight (kg)	66.8 (8.9)	101.2 (11.1)	0.006	60.5 (7.1)	128.3 (16.6)	<0.0001
	Blood Pressure						
	Systolic (mmHg)	112 (8)	127 (9)	0.086	112 (8)	125 (9)	0.011
	Diastolic (mmHg)	70 (5.6)	79 (3.5)	0.048	66 (6)	73 (8)	0.063
	Parity N (%)						
	Primiparous	N/A	N/A	N/A	4 (50)	3 (42.8)	0.736
	Para 1				3 (37.5)	2 (28.1)	
	Para 2				1 (12.5)	2 (28.1)	
	Gestational age at study visit (days)	N/A	N/A	N/A			
	Pregnancy visit 1				132 (7.9)	147 (14.4)	0.03
	Pregnancy visit 2				228 (9.3)	266 (13.0)	0.39
	Gestational at delivery (days)	N/A	N/A	N/A	285 (6.6)	266 (12.9)	0.003
Offspring Characteristics	Offspring gender						
	Male	N/A	N/A	N/A	5	0	0.01
	Female				3	7	
	Infant size						
	Birthweight (g)	N/A	N/A	N/A	3756 (82.8)	3283 (343.7)	0.01
	Birthweight centile				61.5 (14.0)	65.0 (25.4)	0.74
	SDS Score				0.31 (0.4)	0.56 (0.9)	0.52

Table 5-1. Maternal and Neonatal Characteristics

Age, BMI, weight and blood pressure were recorded at ‘booking’ in pregnant subjects, and study visit 1 in non-pregnant subjects. Parity was defined as ‘primiparous’ (no previous pregnancies delivered after 24 weeks), ‘Para 1’ (one previous delivery at 24 weeks), and ‘Para 2’ (two or more previous deliveries after 24 weeks gestation). Birthweight excludes one baby of an obese subject, who was born preterm at 34 weeks

gestation. Data are mean (sd) or N (%).

Key: BMI, body mass index; SDS, standard deviation birthweight score; N/A, not applicable.

5.4.2 Serum Hormone Profiles (08.00h-11.00h and 16.00h-19.00h)

Figure 5-1 shows an example of serum glucocorticoid hormone profiles in non-pregnancy and during the two pregnancy visits, as illustrated by the data collected from the obese subject who was studied in non-pregnancy (two months pre-pregnancy), and during pregnancy at visit 1 (17+2 weeks' gestation), and visit 2 (30+6 weeks' gestation). Levels of cortisol (**Figure 5-1a**), cortisone (**Figure 5-1b**), corticosterone (**Figure 5-1c**) and 11-dehydrocorticosterone (**Figure 5-1d**) were higher in pregnancy than in non-pregnancy and all hormone levels increased with advancing gestation. Profiles of serum cortisol, cortisone, corticosterone and 11-dehydrocorticosterone for all other study participants are shown in **Appendix Figures 1-4**. There were no samples from some subjects for the following reasons: did not attend visit 2 (LP2 n=1, OP2 n=2), delivered before visit 2 (OP2 n=1). With visual assessment of hormone profiles, we observed inter-individual variation in pattern of profiles, but there were no obvious differences in characteristics between women that had particularly quiescent or dynamic profiles. Only one cortisol pulse was detected in the sampling from one subject so serum pulse characteristics are not reported. Interstitial fluid pulses were detected in the profiles of all subjects. Pulse characteristics are demonstrated in **Appendix Table 3**, and key findings are described below.

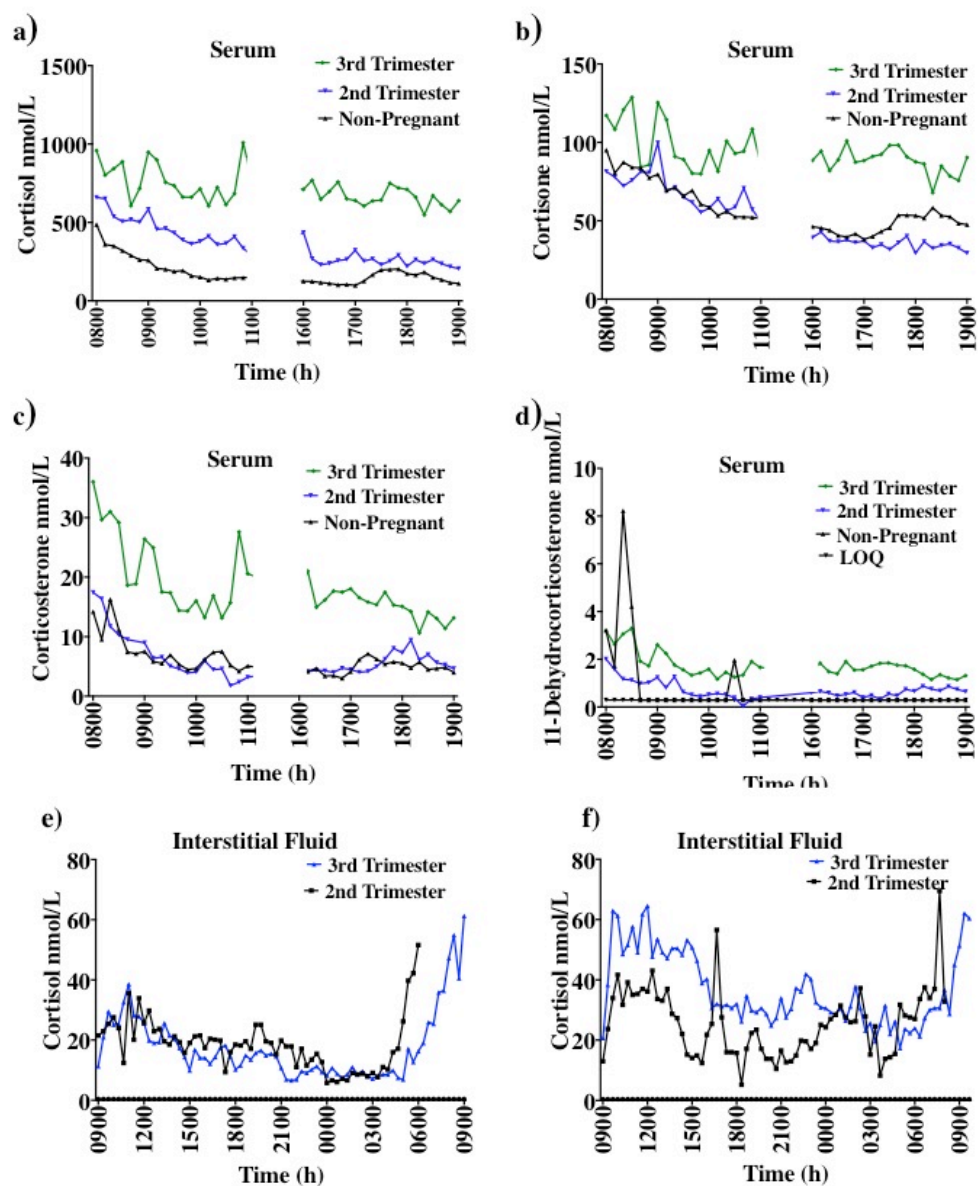


Figure 5-1 a-f. Serum and Interstitial Fluid. Profiles of serum cortisol (1a), cortisone (1b), corticosterone (1c), and 11-dehydrocorticosterone (1d)

Each figure demonstrates the individual hormone profiles of one representative subject who was studied two months pre-pregnancy, at visit 1 (17+2 weeks' gestation) and visit 2 (third trimester (30+6 weeks' gestation)). Interstitial fluid cortisol

profiles from two representative lean subjects obtained at 17+2 and 31 weeks (1e) and 18+3 and 32+3 weeks (1f).

Key: LOQ (limit of quantification)

5.4.3 Serum Cortisol and Cortisone

Fasting serum cortisol and cortisol AUC were significantly higher in pregnancy than non-pregnancy in lean and obese (**Figure 5-2 a-b**). During pregnancy, cortisol AUC increased significantly with advancing gestation in lean, but not in obese (**Figure 5-2 a-b**). Plasma ACTH rose with advancing gestation in both lean and obese groups (**Figure 5-2c**). The mean ‘highest peak’ and ‘lowest trough’ increased significantly with gestation in both lean and obese (**Appendix Table 5-3**).

Serum cortisone AUC was higher in pregnancy than non-pregnancy in both lean and obese. During pregnancy overall cortisone levels rose (**Figure 5-1b**). Cortisone ‘lowest trough’ increased significantly during pregnancy in obese, but not in lean (**Appendix Table 5-3**).

The mean cortisol:cortisone ratio of serum profiles was highest at pregnancy visit 1 (**Table 5-2**). The ratio of the baseline (08.00h) sample was significantly higher in pregnancy than non-pregnancy in obese but not in lean.

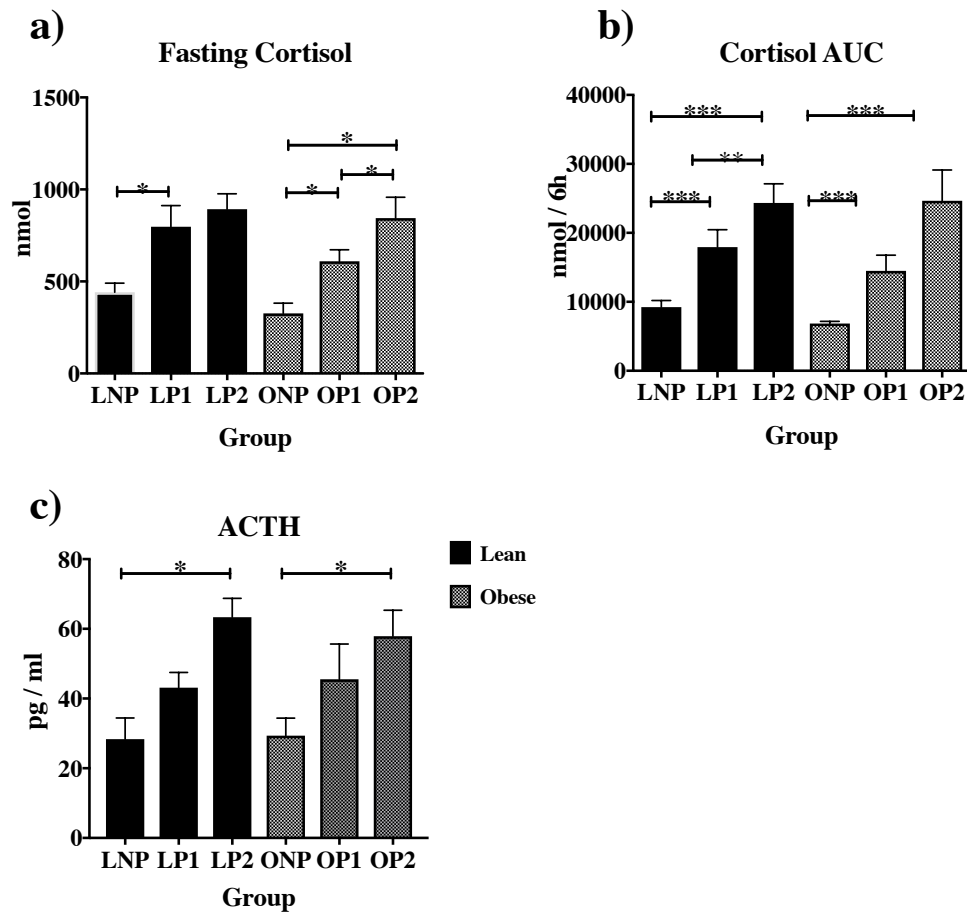


Figure 5-2 (a-c) Fasting serum cortisol (2a) increased with advancing gestation in lean and obese (one-way ANOVA: lean $p=0.017$, obese $p=0.027$), and was significantly higher at OP2 than OP1 (paired t-test $p=0.012$), and at OP2 than ONP (post-hoc Tukey, $p=0.046$). Cortisol AUC of serum profile (2b) increased with advancing gestation in lean and obese (one-way ANOVA: lean $p<0.0001$, obese $p<0.0001$). AUC increased significantly between visit 1 and visit 2 in lean pregnant women (LP1 17937 ± 7141 nM vs LP2 2345 ± 7328 nM, $p=0.001$), but not in obese (OP1 14498 ± 5794 vs OP2 24660 ± 8937 nM, $p=0.06$). Plasma ACTH levels (2c) increased during pregnancy and were significantly higher at pregnancy visit 2 than non-pregnancy in lean ($p=0.017$) and obese ($p=0.045$).

Key: Lean non-pregnant (LNP), lean pregnant visit 1 (LP1), lean pregnant visit 2 (LP2), Obese non-pregnant (ONP), obese pregnant visit 1 (OP1), obese pregnant visit 2 (OP2). Data on graphs are mean (sem). * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

	Lean					Obese				
	LNP	LP1	LP2	p ¹	p ²	ONP	OP1	OP2	p ¹	p ²
Cortisol: Cortisone Baseline sample	4.6 (1.1)	7.9 (4.2)	6.9 (3.3)	0.22	0.002	4.3 (0.7)	6.7 (1.1)	5.7 (1.9)	0.007	0.018
Cortisol: Cortisone Mean overall	3.3 (0.4)	5.8 (3.1)	5.6 (2.1)	0.135	0.004	3.5 (0.4)	5.9 (0.9)	5.3 (1.8)	0.001	0.078
Corticosterone: 11-dehydrocorticosterone Baseline sample	2.5 (1.1)	6.4 (5.6)	5.9 (3.8)	0.439	0.01	8.0 (10.3)	4.2 (3.0)	4.8 (2.5)	0.728	0.129

Table 5-2. Cortisol:Cortisone Ratio

Cortisol: cortisone ratios of the first baseline fasting sample, mean of all samples, mean of first sampling time (08.00h-11.00h) and mean of second sampling time (16.00h-19.00h). One-way ANOVA found that cortisol: cortisone on the first baseline sample and throughout the profile was significantly higher in pregnancy than non-pregnancy in obese but not in lean. The paired t-test showed that during pregnancy, fasting cortisol: cortisone was significantly higher at visit 1 than visit 2 in lean and in obese and that cortisol: cortisone ratio for the whole profile was significantly higher in lean but not in obese. Corticosterone: 11-dehydrocorticosterone was significantly higher at pregnancy visit 1 than visit 2 in lean, but not in obese. The ratio of corticosterone: 11-dehydrocorticosterone was not assessed in whole profiles, as a higher proportion of samples were close to or below the limits of quantification. Data are mean (sd). One-way ANOVA p-value (p¹). Paired t-test of pregnancy visit 1 and visit 2 (p²).

Key: LNP, lean non-pregnant; LP1 lean pregnant visit 1; LP2, lean pregnant visit 2; ONP, obese non-pregnant; OP1, obese pregnant visit 1; OP2, obese pregnant visit 2.

5.4.4 Serum Corticosterone and 11-Dehydrocorticosterone

Serum corticosterone levels were highest during pregnancy visit 2 (**Figure 5-1c**) and ‘highest peak’ increased significantly in obese during pregnancy (**Appendix Table 5-3**). Serum 11-dehydrocorticosterone levels were not significantly different with changing gestation (**Figure 5-1d**) and did not differ between obese and lean. The ratio of corticosterone:11-dehydrocorticosterone of the baseline fasting sample was significantly higher at LP1 than LP2, but was not different in obese (**Table 5-2**).

5.4.5 Interstitial Fluid Cortisol

Interstitial fluid data was available from LNP (n=3), LP1 (n=7), LP2 (n=6), ONP (n=4), OP1 (n=7), OP2 (n=3). There were no samples available from some subjects for the following reasons: technical issues (LP1 n=1, OP2 n=1), participant declined (LP2 n=1), did not attend for visit 2 (LP2 n=1, OP2 n=2), delivered before visit 2 (OP2 n=1). Representative profiles from two subjects are shown in **Figure 5-1 e-f**. Both pulsatility and the morning rise in cortisol were detected in the interstitial fluid sampling (**Figure 5-1 e-f**). Pulses were detected in interstitial fluid samples (**Appendix Table 5-3**). In lean women pulse frequency was consistently around 0.2 pulse/hr (approximately one pulse every 5 hours) and did not differ between lean non-pregnant and pregnant women at visit 1 and visit 2 (LNP: 0.23 ± 0.09 pulses, LP1: 0.20 ± 0.14 pulses, LP2: 0.23 ± 0.13 pulses; $p=0.591$). In obese women, pulse frequency was similar to lean in non-pregnancy (0.22 ± 0.12 pulse/hr). Pulse frequency was significantly lower with advancing gestation in obese women (visit 1: 0.18 ± 0.12 pulse/hr, visit 2: 0.04 ± 0.003 pulse/hr; t-test $p=0.025$). There were no differences in pulse amplitude or mean concentration of the whole profiles with increasing gestation, or between lean and obese.

5.5 Discussion

Using two paradigms of altered HPA axis activity in pregnancy to investigate ultradian rhythms of glucocorticoid hormone secretion, we have demonstrated

changes in serum glucocorticoid levels that occur across gestation and in pregnancies complicated by obesity. Total circulating serum cortisol levels were higher in pregnancy than non-pregnancy in lean and obese, and increased significantly with advancing gestation in lean but not in obese. In addition, through measurement of interstitial fluid cortisol levels we show evidence, for the first time, of tissue cortisol pulsatility in human pregnancy. In obese pregnancy, interstitial fluid pulse frequency was lower with advancing gestation.

The observation that serum cortisol AUC significantly increased during pregnancy in lean women, but not in obese women adds to the previous observations of lower morning fasting cortisol in obese (Stirrat et al., 2016, Berglund et al., 2016), and supports the hypothesis that HPA axis activity is reduced throughout the day in obese pregnancy (Stirrat et al., 2016). These data, together with our finding that interstitial fluid pulse frequency was lower with advancing gestation in obese pregnant women, suggest that an altered ultradian rhythm (characterised by reduced interstitial fluid pulse frequency) may underlie decreased HPA axis in obese pregnancy. In the absence of repeated ACTH sampling, it is not possible to determine the influence of adrenal delay in cortisol release or altered central negative feedback on pulse frequency.

To the best of our knowledge, we are the first to describe circulating corticosterone levels across the day in human pregnancy and in non-pregnant women. Our finding of higher levels of corticosterone in pregnancy than non-pregnancy suggests that like cortisol, the synthesis and release of this hormone is influenced by increased activation of the maternal HPA axis (Duthie and Reynolds, 2013). Unlike cortisone, levels of 11-dehydrocorticosterone did not change significantly with advancing gestation, suggesting that there may be a lesser breakdown of corticosterone than cortisol by 11 β -HSD2 or alternative pathways of corticosterone clearance. For example the ABC-transporters ‘p-glycoprotein’ (P-gp, encoded by *ABCB1*) and ‘multidrug resistant protein-1’ (MRP1, encoded by *ABCC1*) which preferentially export cortisol or corticosterone, respectively are present in the placenta (St-Pierre et al., 2000), and *ABCB1* mRNA expression is reported to be lower in placentas of pregnancies complicated by severe obesity (Mina et al., 2015). Further studies are needed to understand the metabolism of corticosterone in pregnancy.

Our study is the first to measure interstitial fluid cortisol in women, and in human pregnancy. We analysed the pulsatile characteristics of this data using the well-established Cluster algorithm (Veldhuis and Johnson, 1986). This is a statistically-rigorous method that takes into account assay prevision and is not adversely influenced by drifting baseline hormone secretion. Moreover, its model-independent nature means that it is not reliant on a priori assumptions about the system (eg. parameters defined dynamics of hormone secretion or clearance) that we have little information about in lean or obese pregnancy conditions. Using this algorithm, we demonstrated tissue cortisol pulsatility in women, and in human pregnancy. In all women, interstitial fluid cortisol pulse frequency was similar to the findings of a previous study of circulating serum cortisol in pregnancy. Magiakou *et al.*, (Magiakou et al., 1996) used the Detect pulse analysis method (Oerter et al., 1986) to identify cortisol pulses in samples obtained at 30 minute intervals, and observed 2-3 pulses in a 12-hour period in pregnant women between 34-36 weeks gestation. Though the authors acknowledged that the 30 minute sampling may have limited their detection of cortisol pulses, our observations of no serum cortisol pulses over the 6 hour sampling frame in all but one subject, and the similar interstitial fluid cortisol pulsatility to Magiakou *et al.*, 1996 suggests that circulating cortisol pulsatility in pregnancy is decreased compared with the findings of previous studies of circulating cortisol in men (Follenius et al., 1987, Veldhuis et al., 1989) and in rats (Jasper and Engeland, 1991) reporting that pulses occur approximately hourly (Veldhuis et al., 1989, Follenius et al., 1987, Jasper and Engeland, 1991). We sampled serum more frequently (10-minute sampling interval) to increase the likelihood of identifying serum glucocorticoid pulses and conducted our studies with woman in the fasting state to exclude the pulsatile response that has been reported to occur in response to food (Fall et al., 2002). For these reasons the sampling duration was shortened (six hours at each visit) to limit the total blood volume sampled from pregnant women. We can only speculate that this shorter sampling time was not long enough to detect pulses using Cluster.

Studies of the dynamic nature of glucocorticoids in humans are challenging, due to the need to obtain frequent samples. This is particularly pertinent in pregnant women

where there may be ethical restrictions on the volume of blood sampled over the day. However, as our understanding of the role of the ultradian rhythm of cortisol in transcriptional regulation increases, and associations between an altered ultradian rhythm and disease processes are described (Lightman and Conway-Campbell, 2010), there is a need for dynamic studies. This is particularly so in the context of pregnancy, where the links between glucocorticoid exposure *in utero* and adverse fetal outcomes are well described but mechanisms are not well understood. Dynamic studies are needed to investigate the impact of the ultradian rhythm of cortisol on potential targets such as fetal or placental gene expression or function, as a potential underlying mechanism for the abnormal fetal growth (Stewart et al., 1995) and increased risk of cardiovascular disease later in life (Reynolds, 2013) that have been linked to abnormal glucocorticoid exposure.

Strengths of our study include frequent sampling, matched serum and interstitial fluid sampling, sampling at two different points in gestation and use of a robust statistical method for pulse detection and analysis. Obtaining serum samples via a peripheral cannula, and interstitial fluid samples via a microdialysis catheter, meant that frequent sampling could take place without repeated insertion of needles, which would mount a stress response. Another strength is that serum samples were analysed with gold-standard analytical technique of liquid chromatography-tandem mass spectrometry (LC-MS/MS), (Jung et al., 2011) which also allowed the simultaneous detection of multiple analytes from a small volume of sample.

A limitation of our study is that the serum sampling periods were relatively short (two 3-hour study periods), during which only one cortisol pulse was detected in one subject's sampling profile. These durations were selected so that sampling could be performed with subjects fasting, to avoid the known pulsatile response to food (Fall et al., 2002, Stimson et al., 2014), and so that a comparison could be made between morning and evening cortisol levels, whilst considering blood withdrawal restrictions of pregnant women. We acknowledge that mathematical algorithms for hormone pulse analysis have not been previously tested in a pregnant population, where there is an unknown contribution of the placenta to cortisol metabolism and clearance. We could not directly examine placental cortisol metabolism and transfer in this study. We used the ratio of cortisol:cortisone to infer total body 11 β -HSD2 activity, which

we assume in pregnancy is largely reflective of the placenta. The relative reduction in the ratio between visit 1 and visit 2 of pregnancy, suggests that the activity of 11 β -HSD2 increases at the later gestations, thus serving to protect the fetus from exposure to excessively high maternal cortisol levels during the third trimester (Duthie and Reynolds, 2013). Further studies are needed to understand more about the contribution of the placenta to maternal and fetal glucocorticoid exposure.

In conclusion, our study has demonstrated that total day-time circulating maternal cortisol increases with advancing gestation in lean, but not obese women. Further, we have demonstrated the novel finding that tissue cortisol levels are pulsatile during human pregnancy, and that interstitial fluid pulse frequency is significantly lower with advancing gestation in obese pregnancy. This may be an underlying mechanism for the reduced HPA axis activity we previously reported in obese pregnancy (Stirrat et al., 2016). Further studies extending the serum sampling time in order to detect serum pulses would confirm whether or not this finding is consistent in circulating cortisol level. A better understanding of the role of HPA axis dysregulation in adverse pregnancy outcomes may inform us which high-risk pregnancies should be targeted to improve the health of the pregnant woman and the developing baby.

5.6 Appendices

	Molecular Weight g/mol	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>) Quan; Qual	Declustering Potential (V)	Collision energy (V) Quan; Qual	Cell exit potential (V) Quan; Qual
ANALYTES						
Cortisol	362.2	363.2	121, 77	131	29, 101	14, 14
Cortisone	360.2	361.2	77; 163.2	166	99; 11	36; 20
Corticosterone	346.1	347.1	121.1; 97.1	66	69; 69	8; 8
11-dehydrocorticosterone	344.1	345.1	121.2; 90.9	51	33; 71	8; 10
INTERNAL STANDARDS						
D ₄ -cortisol	366.5	367.0	121; only one	121	25	20
Epi-cortisol	362.5	363.2	121; only one	131	29	14
Epi-corticosterone	340.1	347.1	121; only one	66	69	8

Appendix Table 5-1. Mass spectral conditions for analysis of analytes and internal standards by positive ion electrospray ionisation

Key: Quan, quantifier ion; Qual, qualifier ion; V, volts

	Target Concentration (ng/200 µL)	Intra-assay (<i>n</i> =6)			Inter-assay (<i>n</i> =6)		
		Concentration (ng/200 µL): mean (SD)	Precision (% RSD)	Accuracy (%)	Concentration (ng/200 µL): mean (SD)	Precision (% RSD)	Accuracy (%)
Cortisol	Low (2.5)	2.4 (0.2)	9.0	96	2.4 (0.4)	15.9	97
	Mid (100)	102.1 (5.4)	5.3	102	99.2 (9.8)	9.8	99
	High (200)	196.9 (6.0)	3.0	98	195.3 (13.5)	6.9	98
Cortisone	Low (2.5)	2.2 (0.2)	11.0	87	2.7 (0.5)	18.3	107
	Mid (100)	95.9 (6.9)	7.2	96	48.5 (3.2)	6.6	97
	High (200)	212.9 (13.7)	6.4	106	198.4 (4.2)	2.1	99
Corticosterone	Low (0.1)	0.1 (0.005)	4.7	107	0.1 (0.01)	10.7	100
	Mid (10)	9.5 (0.07)	7.0	95	10.0 (1.1)	10.9	100
	High (25)	25.1 (0.7)	2.8	100	25.3 (1.7)	6.6	101
11-Dehydrocorticosterone	Low (0.1)	0.11 (0.0)	14.4	85	0.10 (0.0)	7.3	104
	Mid (15)	16.1 (2.0)	12.5	107	14.9 (1.1)	7.5	100
	High (25)	24.3 (2.6)	10.6	97	26.1 (2.5)	9.7	105

Appendix Table 5-2. Intra-assay and inter-assay precision and accuracy, obtained from aqueous standards as a surrogate matrix.

	Hormone Characteristics	Group						p-value	
		LNP	LP1	LP2	ONP	OP1	OP2	Lean	Obese
Serum	Baseline fasting								
	Cortisol	440.9 (85.6)	797.4 (325.2)	892.6 (221.2)	326.3 (112.0)	759.9 (290.2)	840.5 (128.8)	0.017	0.027
	Highest peak (nmol/L)								
	Cortisol	347.0 (94.5)	643.1 (314.3)	859.9 (266.3)	215.6 (33.2)	569.8 (294.5)	600.9 (200.5)	0.017	0.013
	Cortisone	109.1 (40.6)	118.1 (37.4)	151.2 (68.1)	73.4 (13.6)	160.2 (200.9)	166.3 (67.8)	0.342	0.311
	Corticosterone	16.2 (15.1)	32.1 (29.9)	31.1 (11.3)	8.5 (10.1)	18.5 (15.4)	60.5 (36.3)	0.418	0.015
	11-dehydrocorticosterone	33.5 (46.0)	7.9 (7.6)	9.2 (9.3)	4.9 (2.9)	6.1 (3.7)	13.3 (9.7)	0.469	0.269
	Lowest trough (nmol/L)								
	Cortisol	136.5 (58.6)	230.5 (98.8)	353.9 (104.5)	92.6 (28.8)	343.3 (189.9)	234.0 (80.4)	<0.0001	0.035
	Cortisone	48.7 (5.8)	48.5 (11.4)	68.4 (12.6)	73.4 (13.6)	45.9 (20.6)	74.8 (12.2)	0.002	0.022
Interstitial Fluid	Corticosterone	3.4 (4.4)	4.5 (5.1)	4.7 (4.6)	3.6 (2.7)	2.0 (2.1)	4.1 (4.7)	0.992	0.642
	11-dehydrocorticosterone	0.3 (0.0)	3.8 (6.7)	3.8 (7.4)	1.0 (0.8)	0.7 (0.8)	0.9 (0.8)	0.525	0.833
	Profile AUC (nmol/L)								
	Cortisol	9231.8	17937.1(7141.6)	24345.7 (7328.2)	6854.9 (628.4)	14498.6 (5974.1)	24660.1 (8937.4)	<0.0001	<0.0001
	Cortisone	(1681.2)	3075.9 (753.7)	4493.8 (1131.0)	1966.7 (270.0)	2450.2 (1022.8)	4708.9 (1124.1)	0.004	0.019
	Corticosterone	2791.8 (289.6)	577.0 (456.3)	603.9 (349.2)	603.9 (349.2)	297.9 (185.5)	736.4 (307.0)	0.157	0.081
	11-dehydrocorticosterone	419.6 (237.9)	195.8 (281.3)	250.1 (330.6)	47.1 (41.4)	47.2 (30.6)	107.8 (73.7)	0.457	0.564
	Pulse frequency (pulse/hr)	0.23 (0.09)	0.202 (0.14)	0.23 (0.13)	0.22 (0.12)	0.18 (0.12)	0.04 (0.003)	0.591	0.065
	Pulse height (amplitude) (ug/dL)	0.002 (0.0007)	0.0032 (0.002)	0.0023 (0.002)	0.0016 (0.002)	0.0035 (0.002)	0.0022 (0.002)	0.541	0.478
	Mean concentration (ug/dL)	0.34 (0.26)	0.57 (0.28)	0.57 (0.43)	0.23 (0.18)	0.58 (0.25)	0.23 (0)	0.835	0.111

Appendix Table 5-3. Serum cortisol, cortisone, corticosterone, 11-dehydrocorticosterone in lean and obese pregnant and non-pregnant subjects.

Appendix Table 5-3. (cont.)

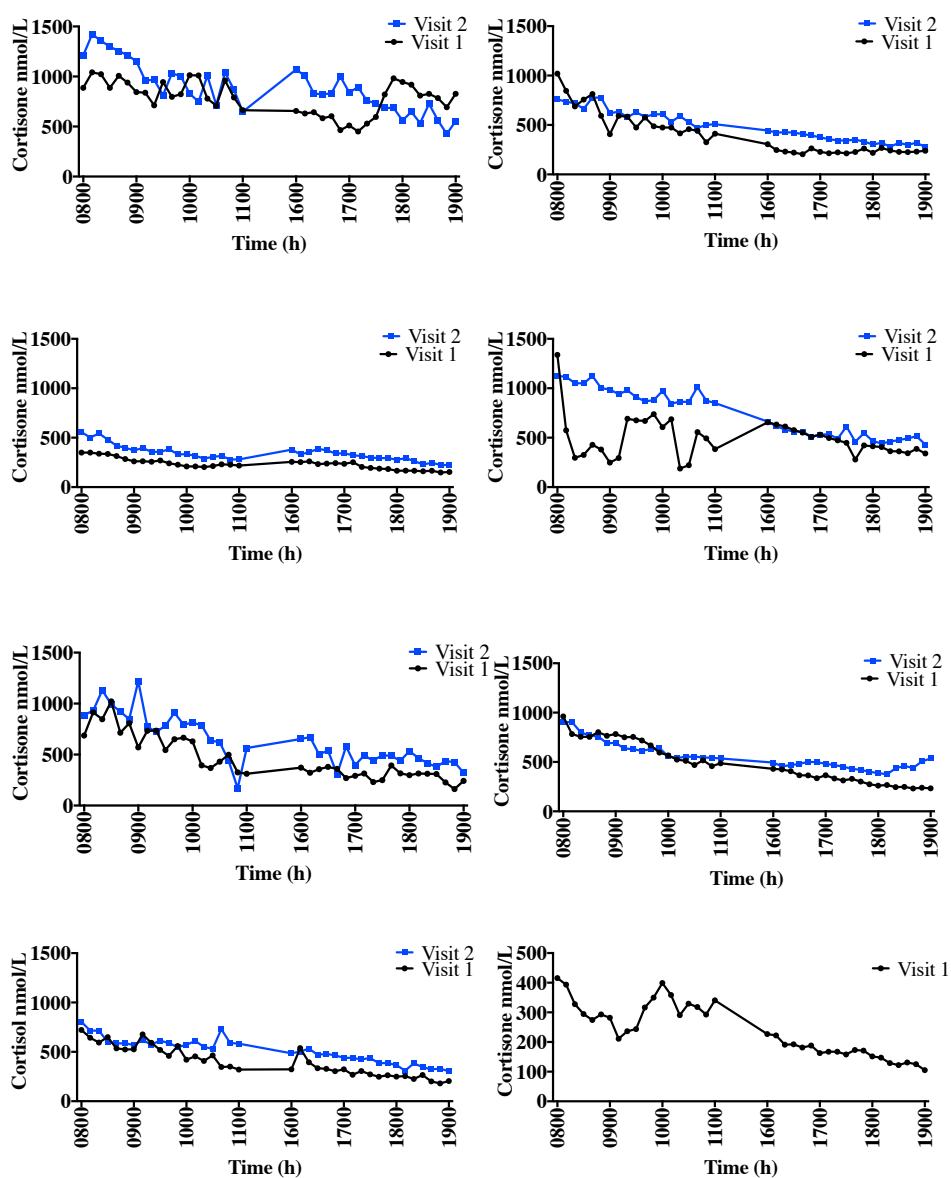
Data are mean (sd). P-values are from one-way ANOVA comparing variables in non-pregnancy, pregnancy visit 1 and pregnancy visit 2, or from t-test comparing change with advancing gestation of pregnancy (*).

Limit of detection in serum were as follows: cortisol (34.5 nmol/L), cortisone (34.7 nmol/L), corticosterone (1.4 nmol/L) and 11-dehydrocorticosterone (1.5 nmol/L). Limits of detection of interstitial fluid cortisol was 0.015 ug/dL.

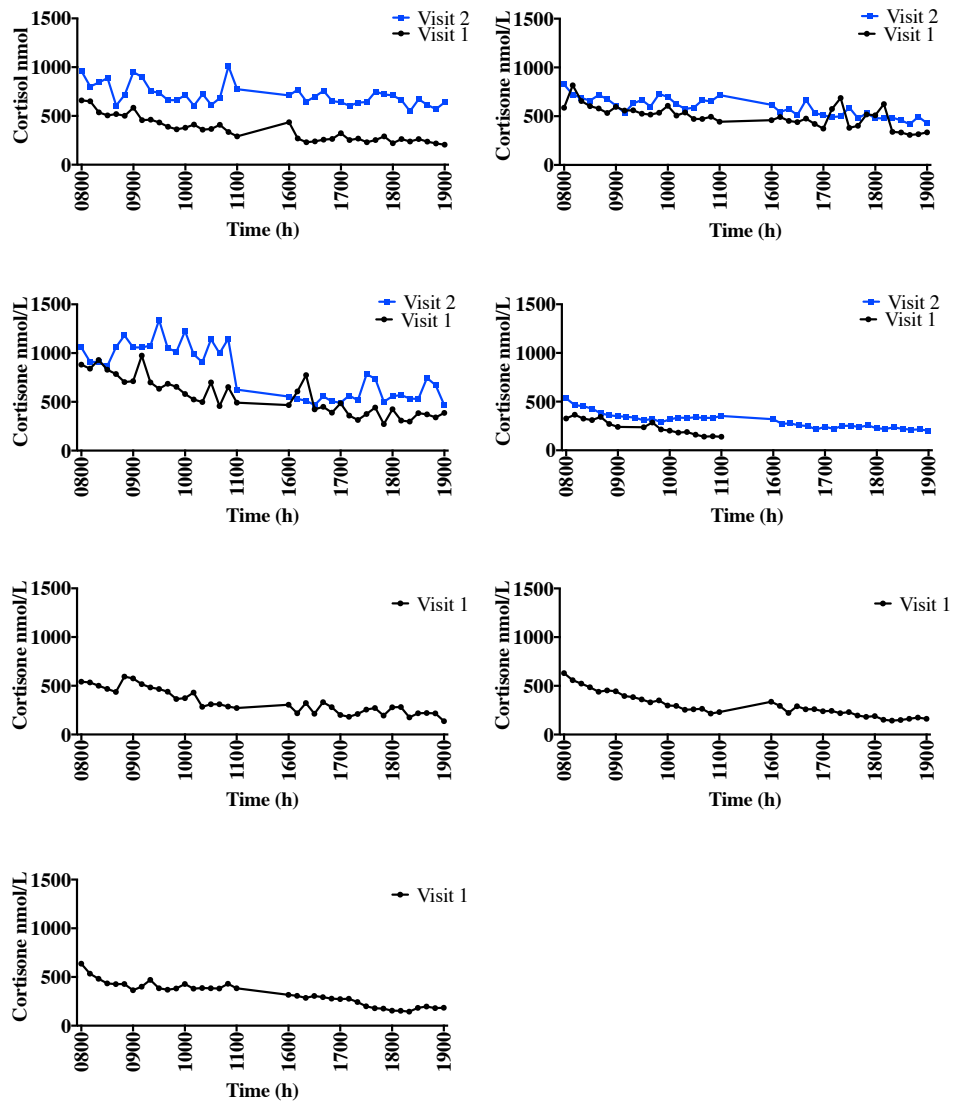
Key: LNP, Lean non-pregnant; LP1, lean pregnant visit 1; LP2 lean pregnant visit 2; ONP, obese non-pregnant; OP1, obese pregnant visit 1; OP2, obese pregnant visit 2.

Appendix Figure 5-1 Serum cortisol profiles from visit 1 (black line) and visit 2 (blue line) in lean pregnant (a) and obese pregnant (b); and from single study visit in lean non-pregnant (c) and obese non-pregnant (d).

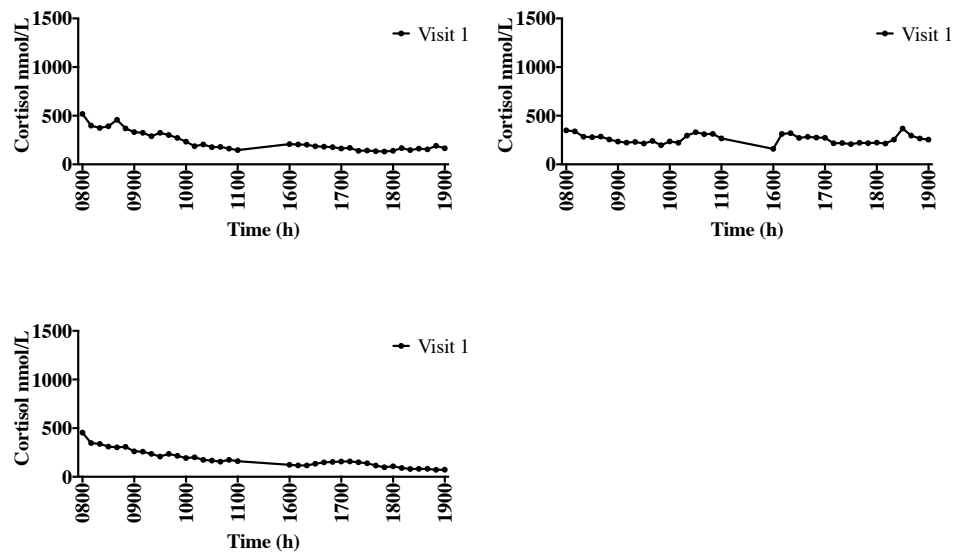
a) Lean Pregnant Cortisol



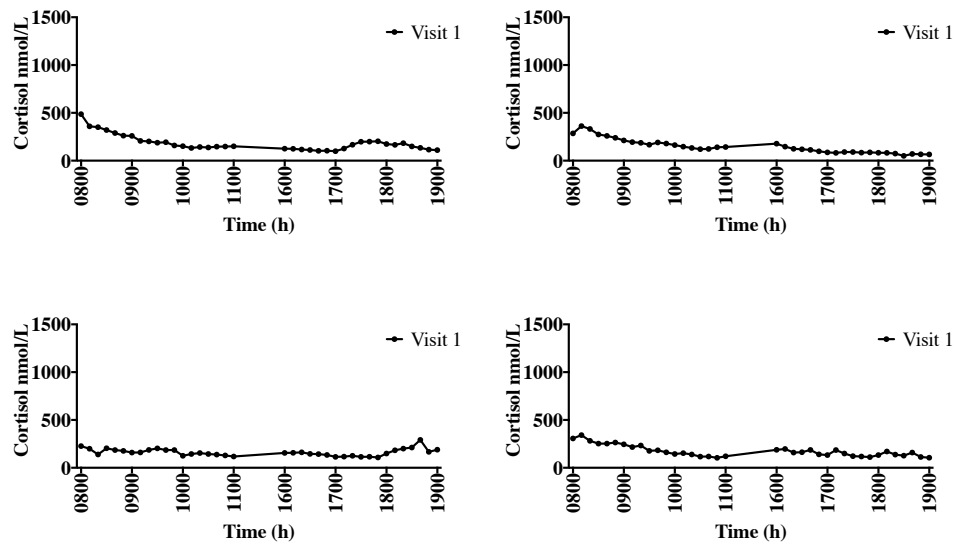
b) Obese Pregnant Cortisol



c) Lean Non-Pregnant Cortisol

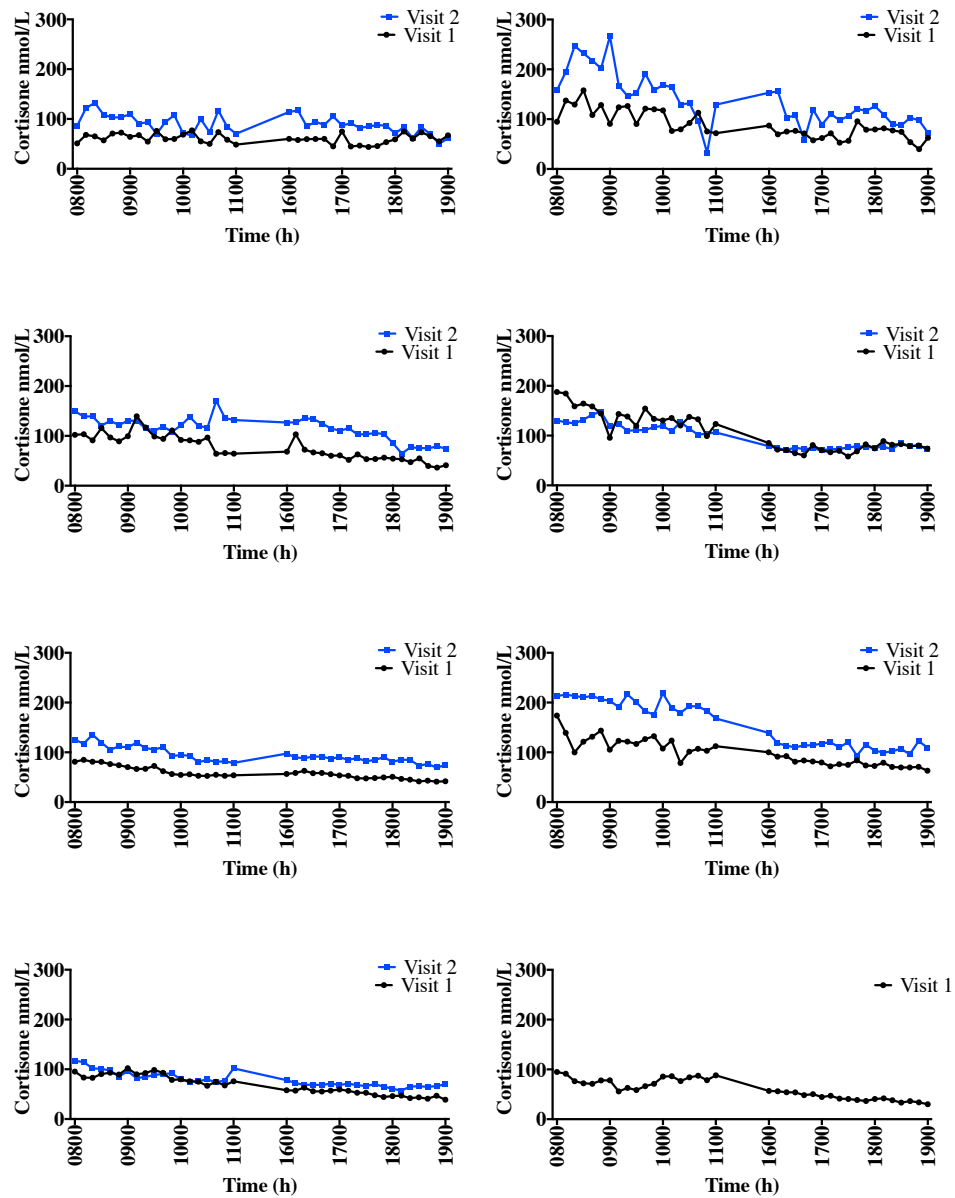


d) Obese Non-Pregnant Cortisol

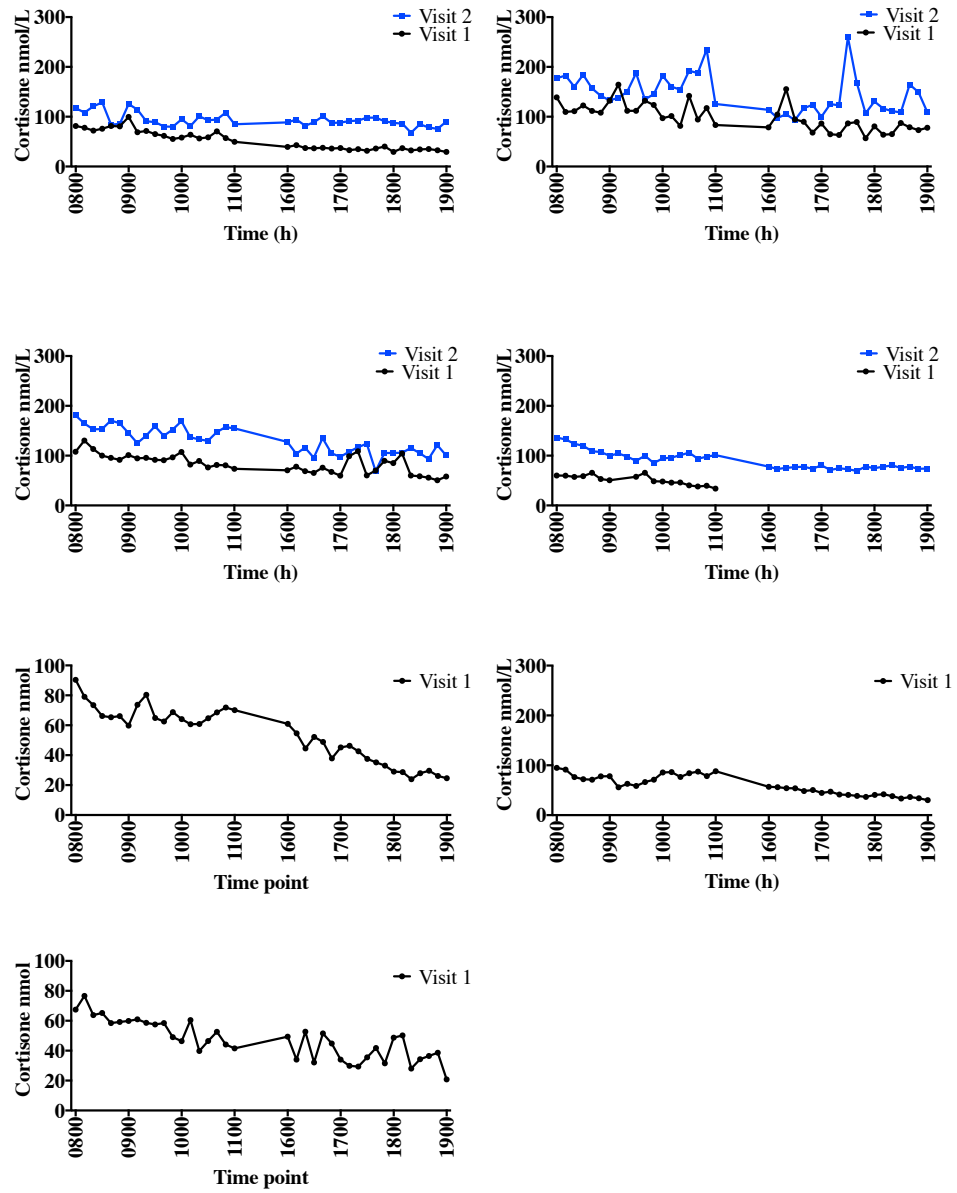


Appendix Figure 5-2. Serum cortisol profiles from visit 1 and visit 2 in lean pregnant (a), obese pregnant (b), lean non-pregnant (c) and obese non-pregnant (d).

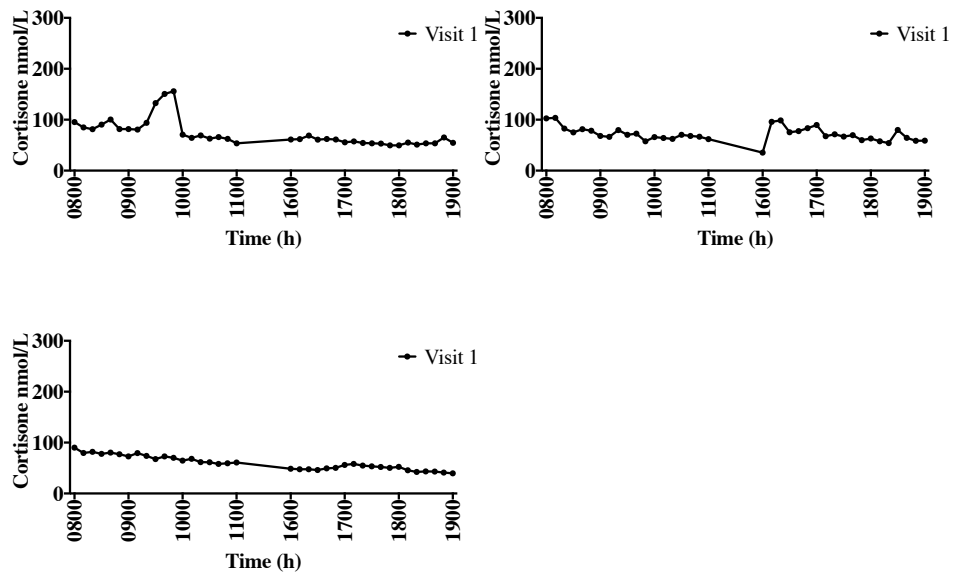
a) Lean Pregnant Cortisone



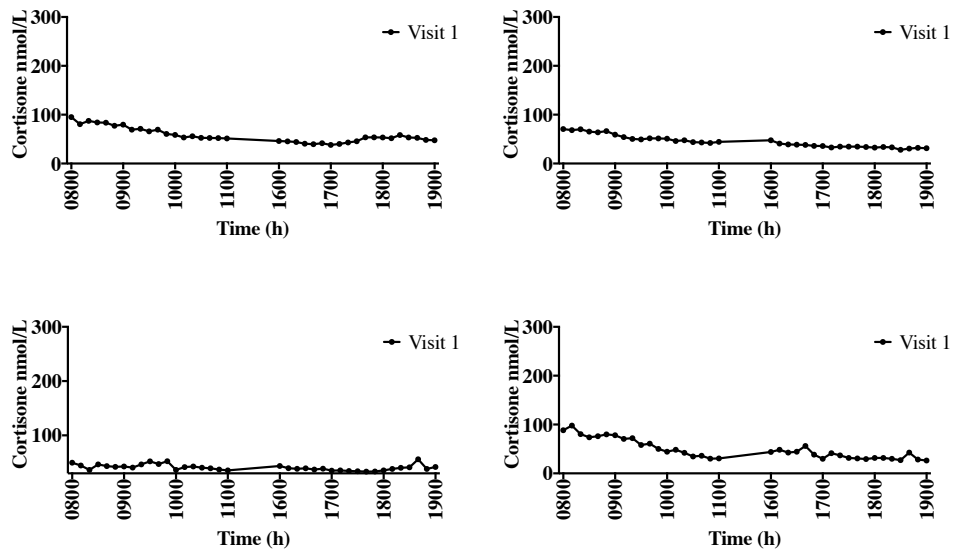
b) Obese Pregnant Cortisone



c) Lean Non-Pregnant Cortisone



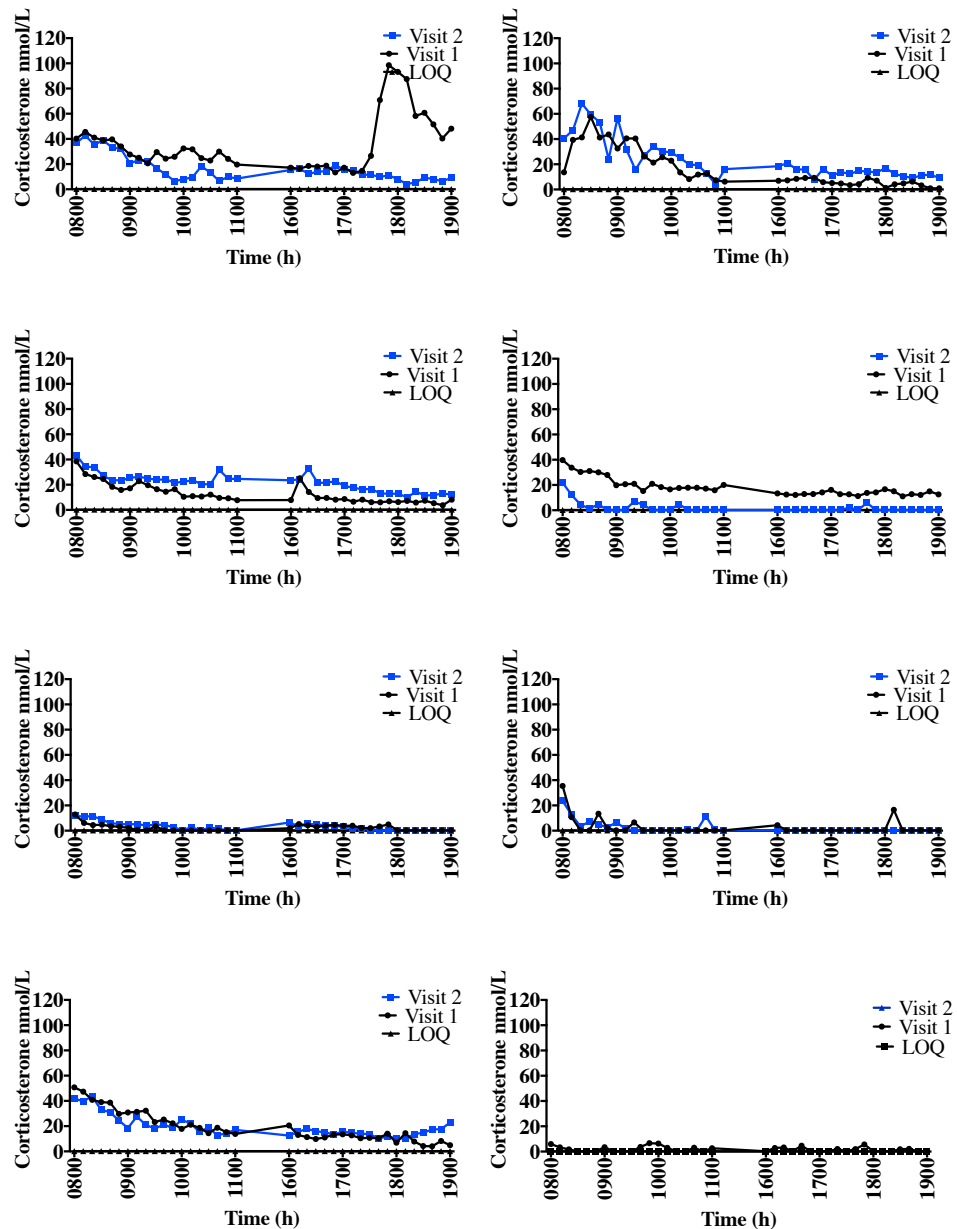
d) Obese Non-Pregnant Cortisone



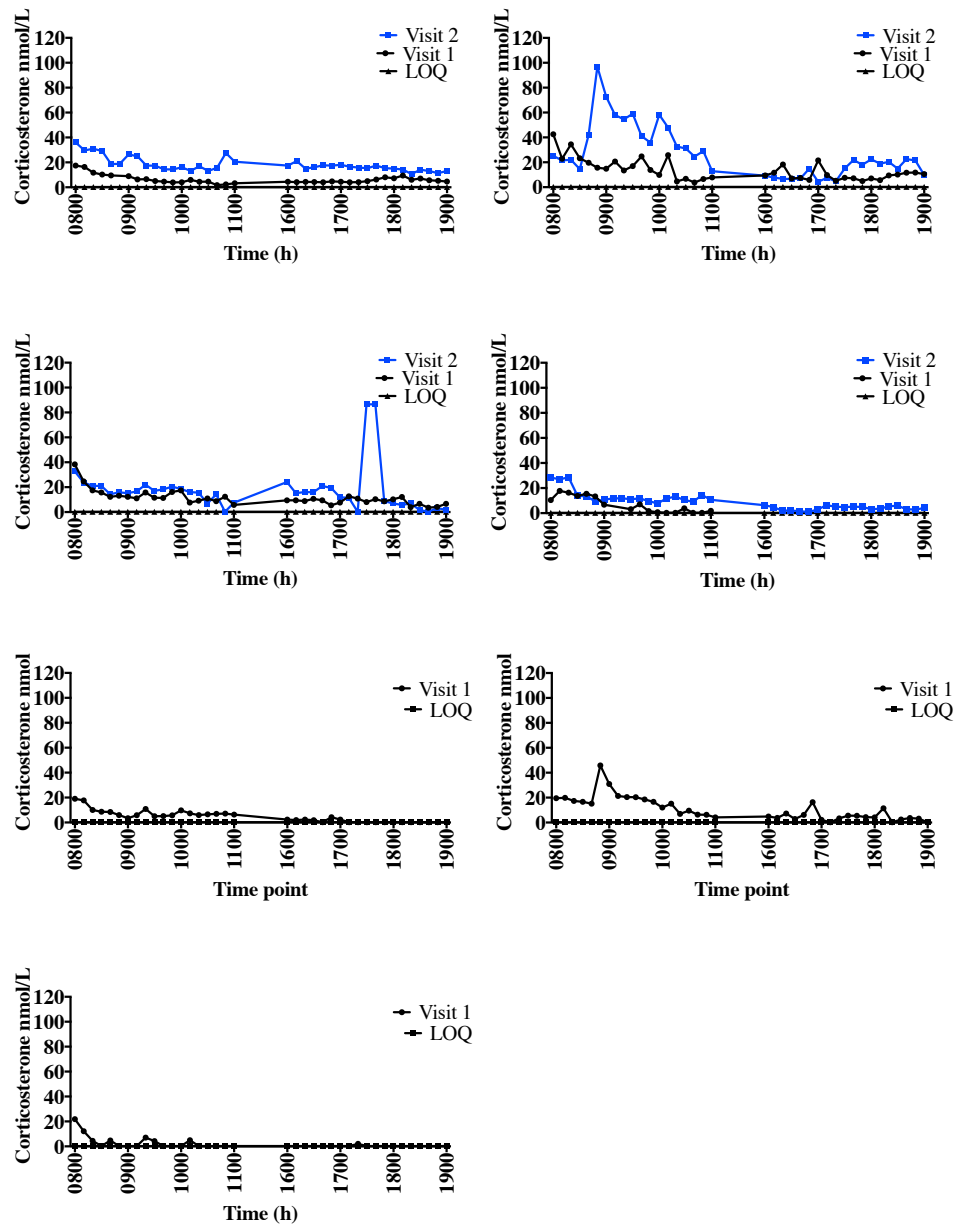
Appendix Figure 5-3. Serum corticosterone profiles from visit 1 and visit 2 in lean pregnant (a), obese pregnant (b), lean non-pregnant (c) and obese non-pregnant (d).

Key: LOQ (limit of quantification; 0.29 nmol/L)

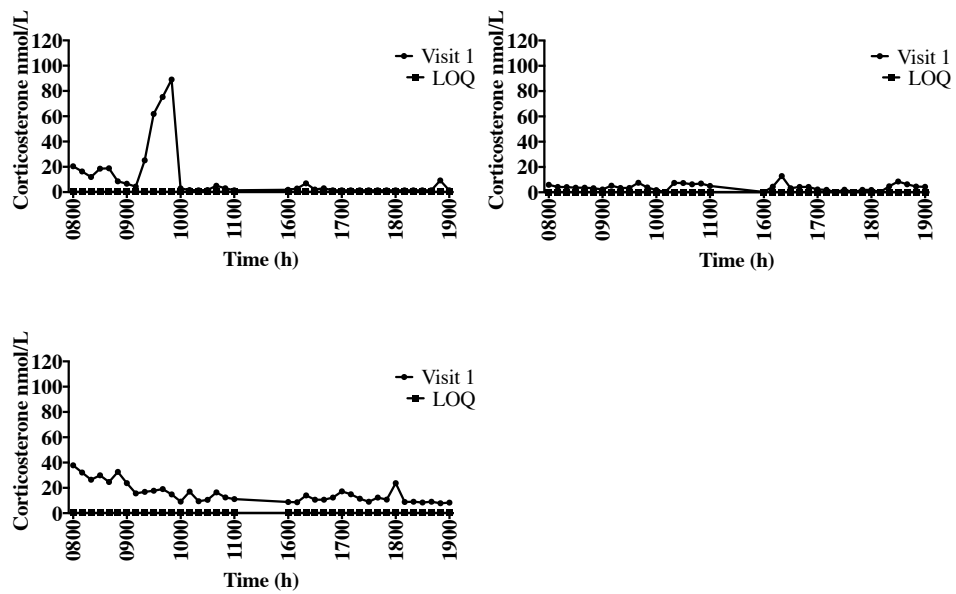
a) Lean Pregnant Corticosterone



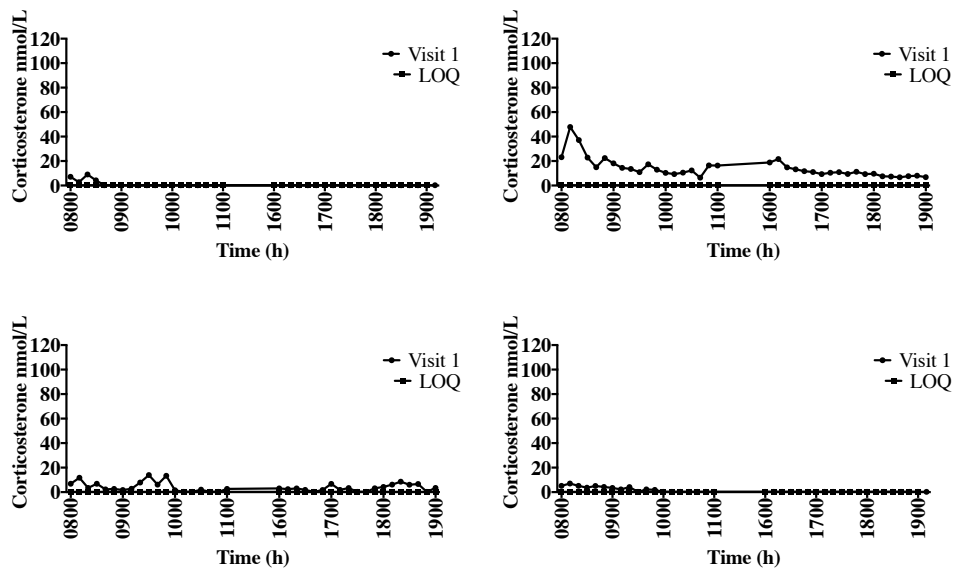
b) Obese Pregnant Corticosterone



c) Lean Non-Pregnant Corticosterone

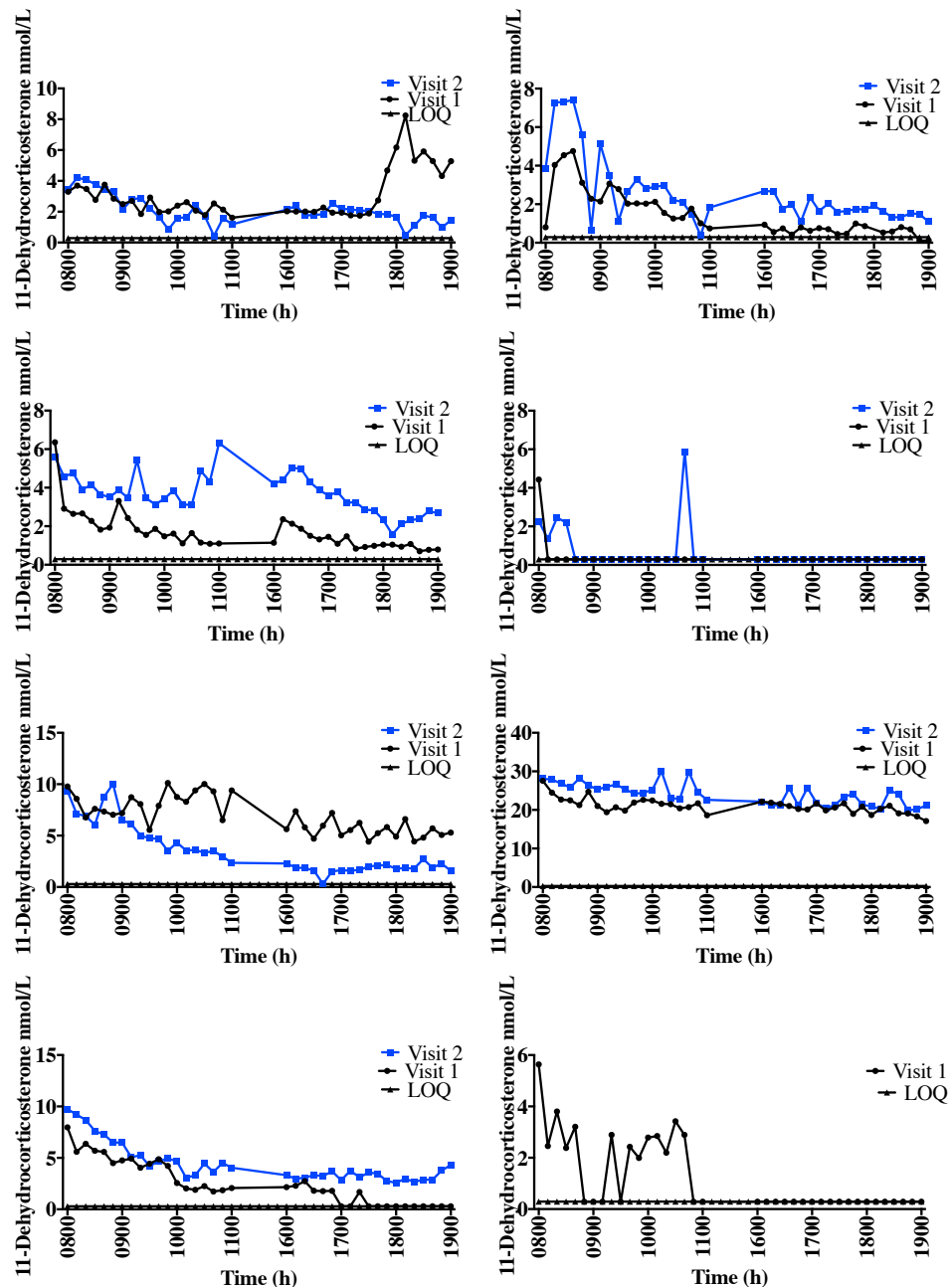


d) Obese non-pregnant corticosterone

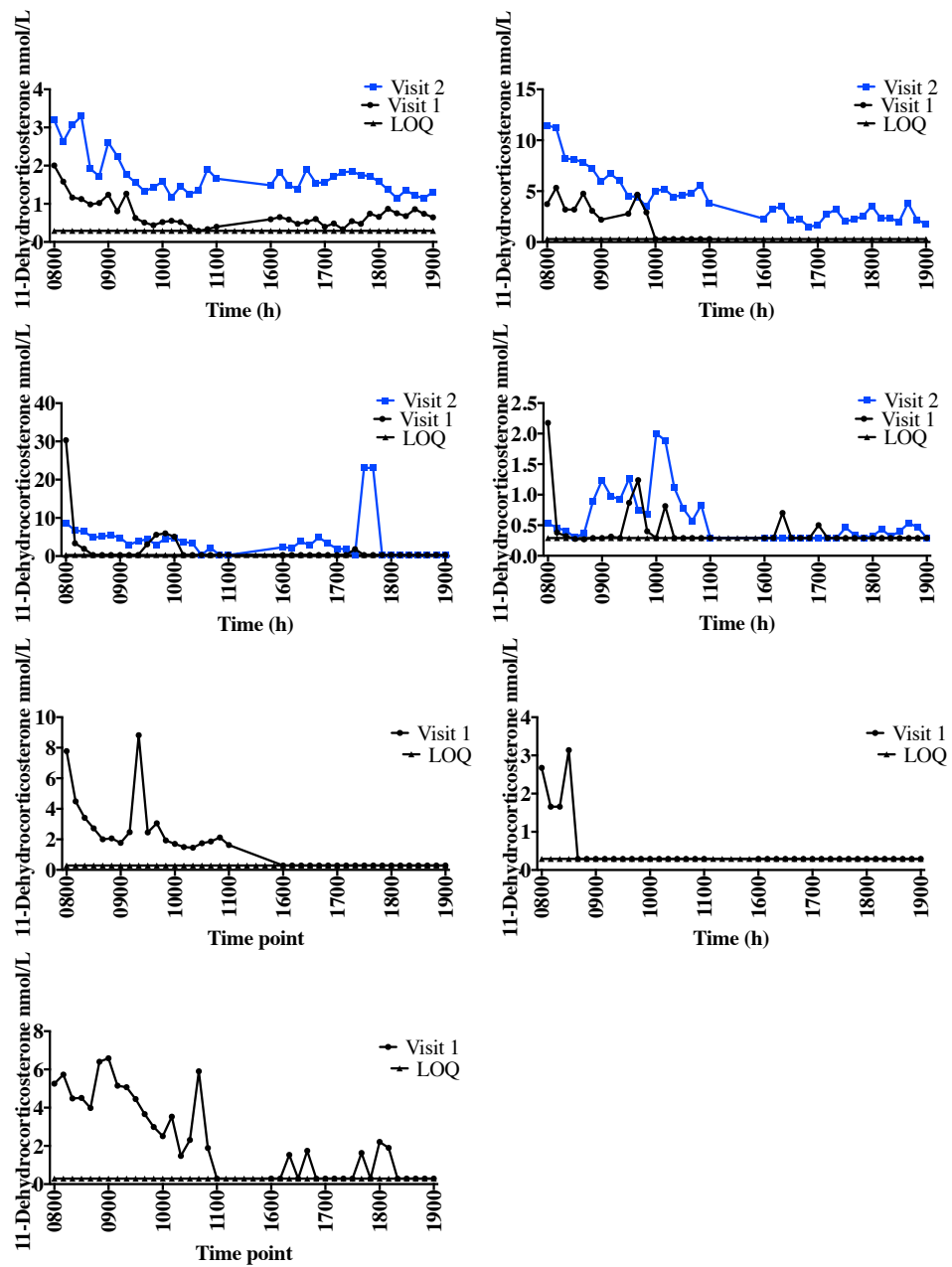


Appendix Figure 5-4. Serum 11-dehydrocorticosterone profiles from visit 1 and visit 2 in lean pregnant (a), obese pregnant (b), lean non-pregnant (c) and obese non-pregnant (d). **Key:** LOQ (limit of quantification; 0.29 nmol/L)

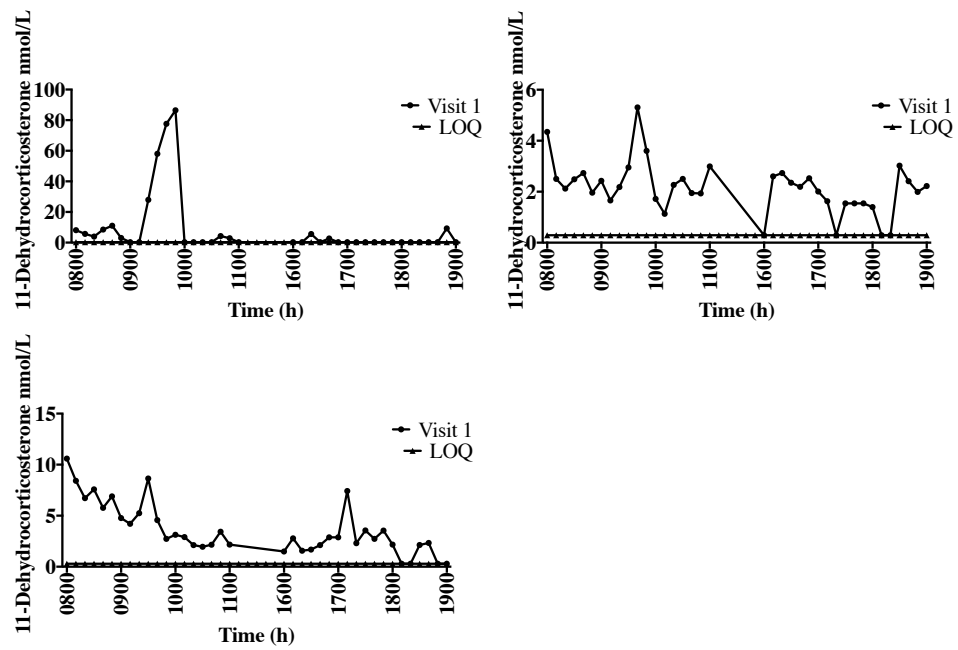
a) Lean pregnant 11-dehydrocorticosterone



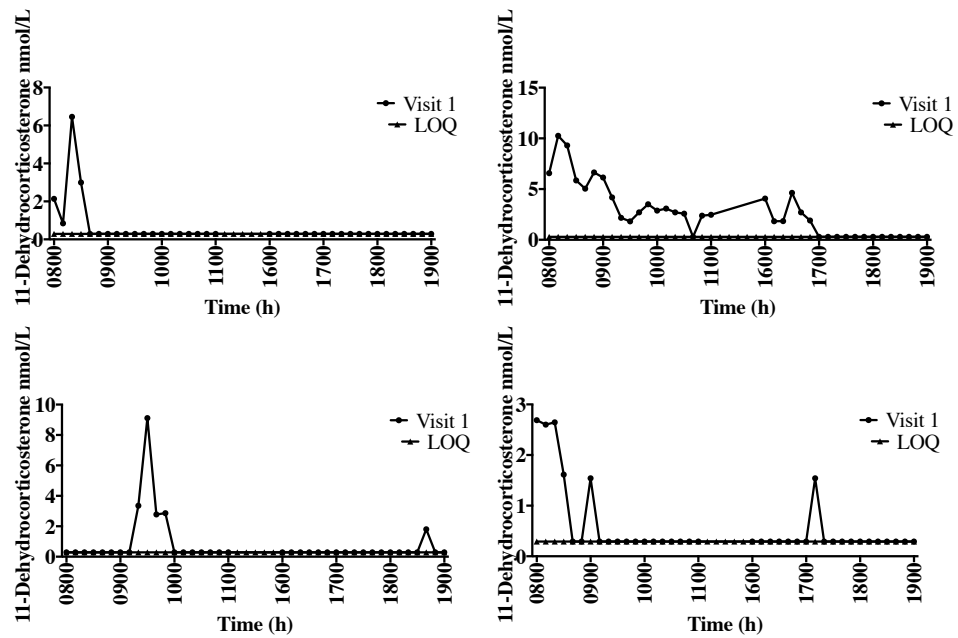
b) Obese pregnant 11-dehydrocorticosterone



c) Lean Non-Pregnant 11-dehydrocorticosterone

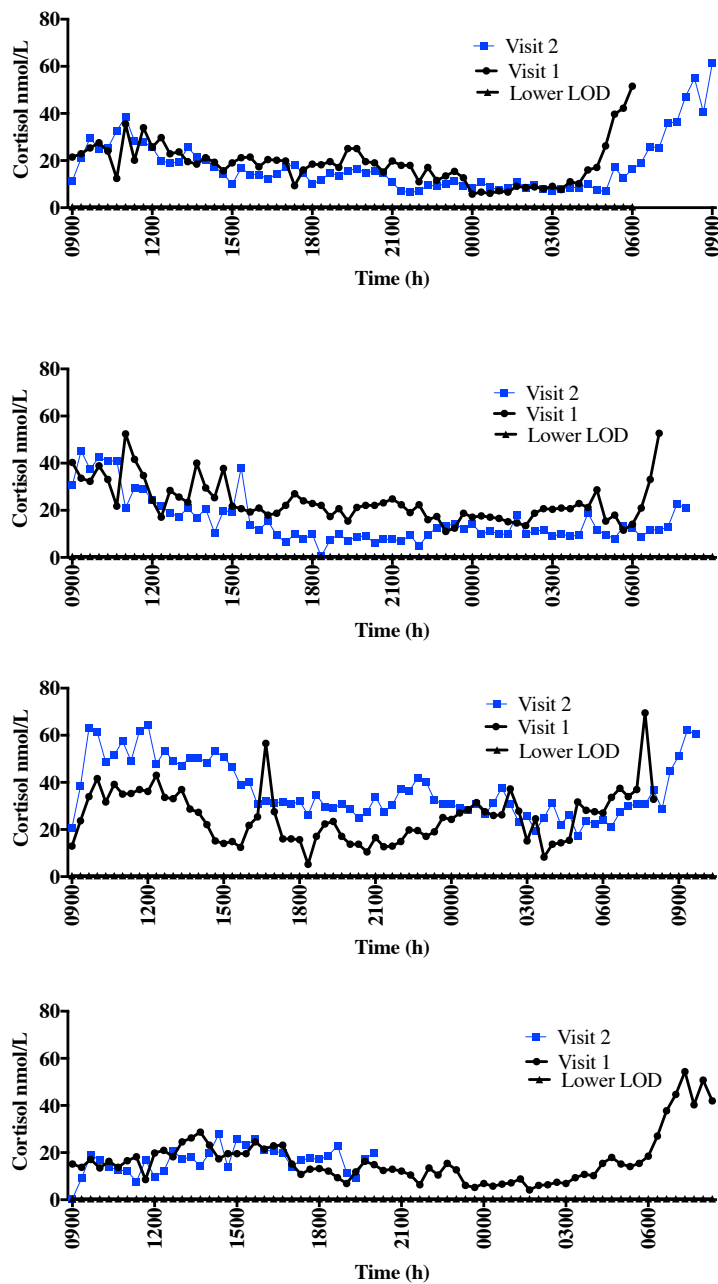


d) Obese Non-Pregnant 11-dehydrocorticosterone

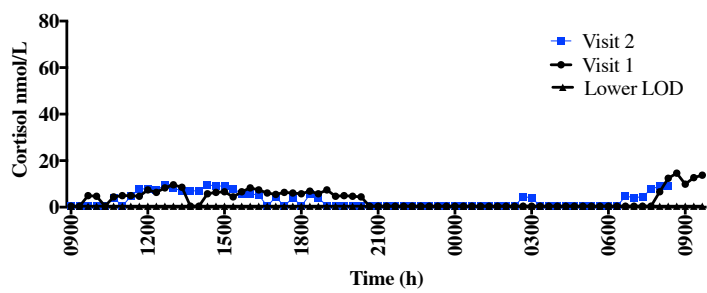
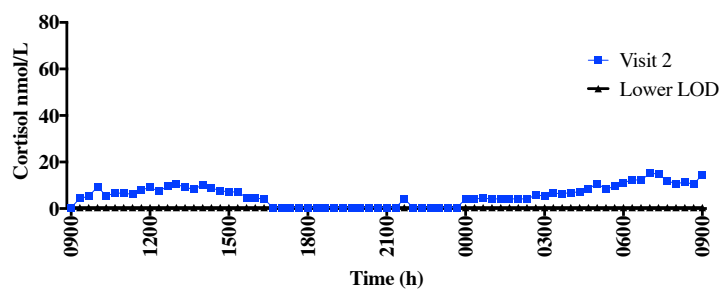
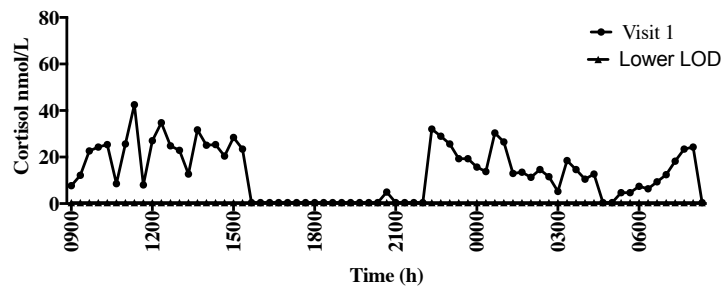


Appendix Figure 5-5. Interstitial fluid cortisol profiles from visit 1 and visit 2 in lean pregnant (a), obese pregnant (b), lean non-pregnant (c) and obese non-pregnant (d). **Key:** LOQ (limit of quantification; 0.41 nmol/L)

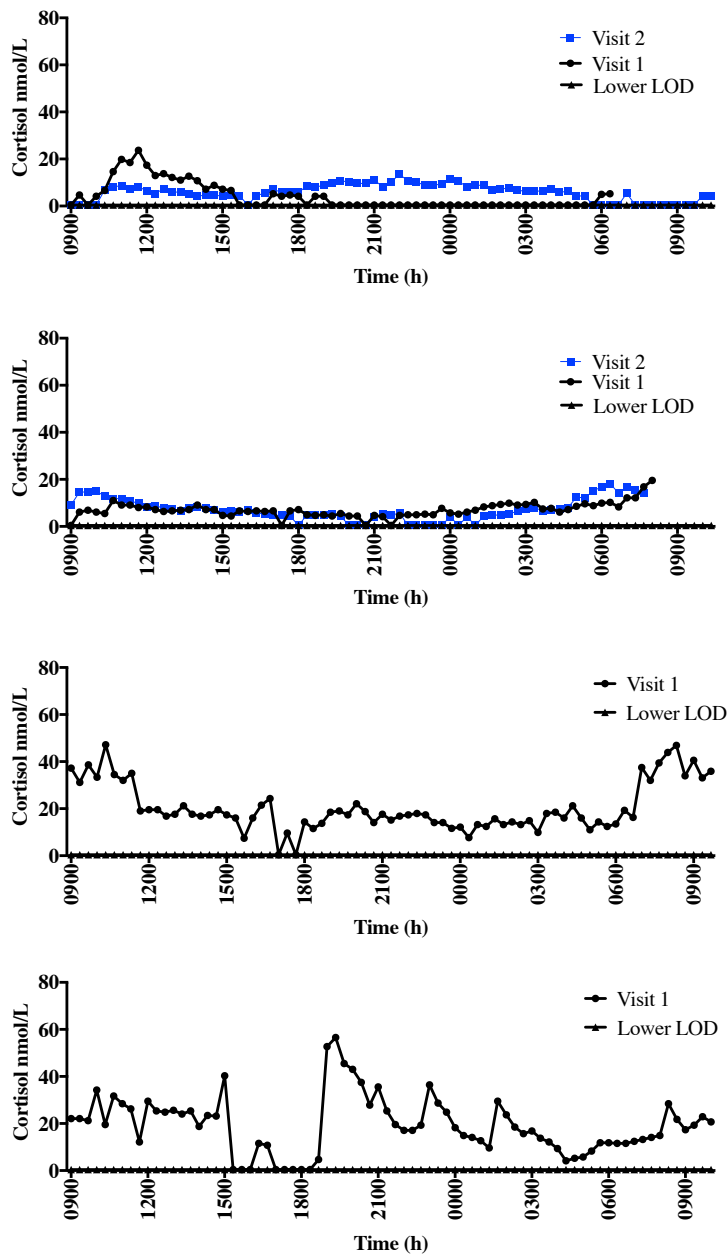
a) Lean pregnant



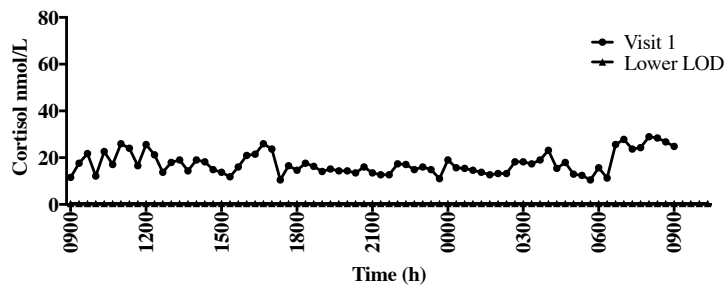
a) Lean pregnant (cont.)



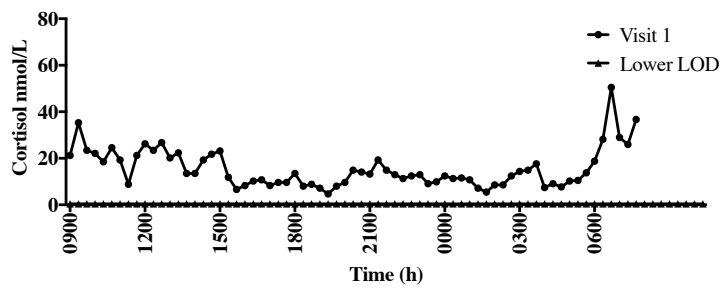
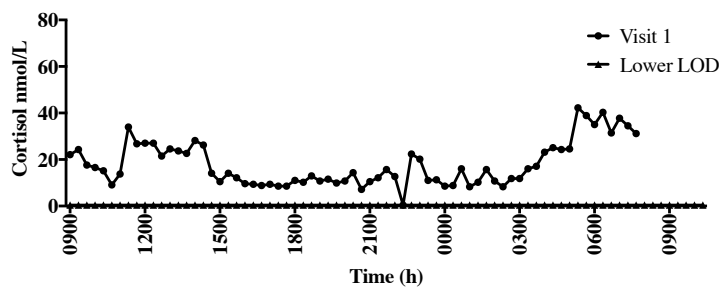
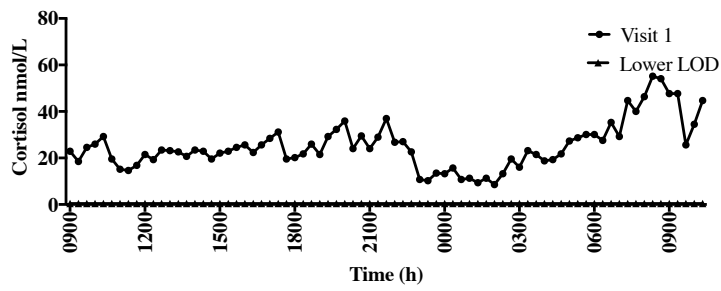
b) Obese pregnant



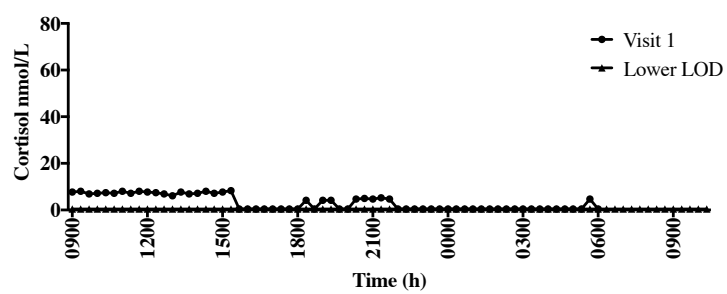
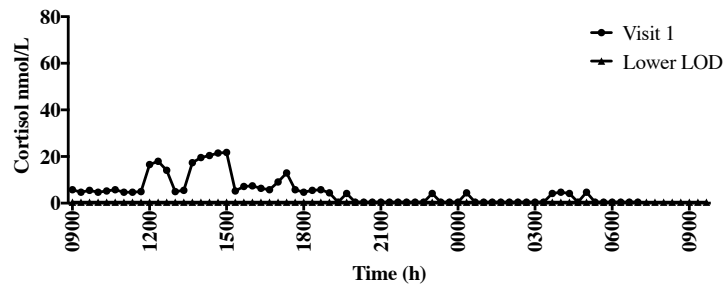
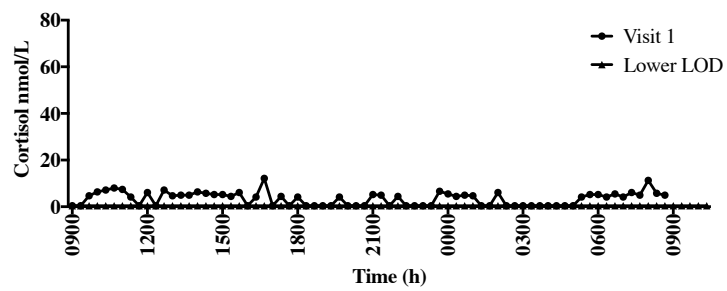
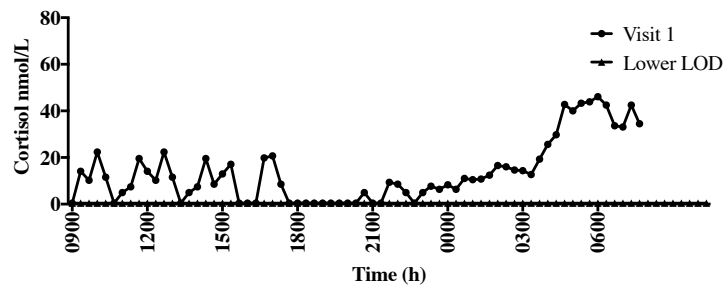
b) Obese pregnant (cont.)



c) Lean Non-Pregnant



d) Obese Non-Pregnant



5.7 Chapter Conclusion

The work in **Chapter 5** examined the effect of increased maternal BMI on glucocorticoid hormones. The findings of this study show for the first time that cortisol is pulsatile in interstitial fluid. Further, the frequency of cortisol pulses in interstitial fluid decreased with advancing gestation in obese pregnancy. An altered ultradian rhythm of cortisol in obese pregnancy may influence overall glucocorticoid exposure, and may be a mechanism underlying the decreased HPA axis activity described in **Chapters 3 and 4**.

It was acknowledged in **Chapters 3-5** that the contribution of the placenta to cortisol transfer and metabolism is not well understood, and may have a significant influence on maternal and fetal cortisol levels during pregnancy. **Chapter 6** relates to laboratory studies investigating placental cortisol transfer and metabolism.

Chapter 6

Transfer and Metabolism of Cortisol by the Isolated Perfused Human Placenta

The following materials have been accepted for publication in the Journal of Clinical Endocrinology and Metabolism under a similar title by Laura I Stirrat (LS), Bram G Sengers (BS), Jane E Norman (JN), Natalie ZM Homer (NH), Ruth Andrew (RA), Rohan M Lewis (RL) and Rebecca M Reynolds (RR).

LS designed the study, conducted the placental perfusion experiments and laboratory analysis, interpreted data and wrote the first draft of the manuscript. BG designed the study, conducted the computational modeling, interpreted data and wrote the manuscript. JN interpreted data. NH and RA advised with laboratory assay development and data interpretation. RL designed the study, conducted computational modeling, interpreted data and wrote the manuscript. RR designed the study, interpreted data and wrote the manuscript. All authors provided critical insight for the manuscript.

In summary, this work demonstrated that the placenta presented both metabolic and physical barriers to cortisol transfer. This challenges the concept that maternal cortisol diffuses freely across the placenta and confirm that 11 β -HSD2 acts as a major ‘barrier’ to cortisol transfer to the fetus.

6.1 Abstract

Context: Fetal overexposure to glucocorticoids *in utero* is associated with fetal growth restriction and is postulated to be a key mechanism linking suboptimal fetal growth with cardiovascular disease in later life.

Objective: To develop a model to predict maternal-fetal glucocorticoid transfer. We hypothesised placental 11 β -HSD2 would be the major rate-limiting step in maternal cortisol transfer to the fetus.

Design: We used a deuterated cortisol tracer in the *ex vivo* placental perfusion model, in combination with computational modeling, to investigate the role of interconversion of cortisol and its inactive metabolite cortisone on transfer of cortisol from mother to fetus.

Participants: Term placentas were collected from five women with uncomplicated pregnancies, at elective Caesarean delivery.

Intervention: Maternal artery of the isolated perfused placenta was perfused with D4-cortisol.

Main Outcome Measures: D4-cortisol, D3-cortisone and D3-cortisol were measured in maternal and fetal venous outflows.

Results: D4-cortisol, D3-cortisone and D3-cortisol were detected and increased in maternal and fetal veins as the concentration of D4-cortisol perfusion increased. D3-cortisone synthesis was inhibited when 11 β -HSD activity was inhibited. At the highest inlet concentration only 3.0% of the maternal cortisol was transferred to the fetal circulation, while 26.5% was metabolised and 70.5% exited via the maternal vein. Inhibiting 11 β -HSD activity increased the transfer to the fetus to 7.3% of the maternal input, while 92.7% exited via the maternal vein.

Conclusions: Our findings challenge the concept that maternal cortisol diffuses freely across the placenta and confirm that 11 β -HSD2 acts as a major 'barrier' to cortisol transfer to the fetus.

6.2 Introduction

Cortisol, the principal circulating glucocorticoid hormone in humans, is essential for normal fetal development and tissue maturation. Fetal overexposure to glucocorticoids *in utero* is associated with intrauterine growth restriction (Stewart et al., 1995), and is postulated to be a key mechanism linking suboptimal fetal growth with increased risk of cardiovascular disease in later life (Reynolds, 2013). Better knowledge of the factors regulating cortisol transfer to the fetus is essential to understand the pathophysiology of fetal growth restriction and is also relevant for prescribing of antenatal steroids which are widely used in clinical management of women at threat of pre-term birth.

Maternal circulating cortisol levels rise dramatically during pregnancy (Jung et al., 2011). Although glucocorticoids are lipophilic and thus are believed to freely cross the placenta, fetal cortisol levels are 5 to 10-fold lower than maternal levels (Beitins et al., 1973) due to the activity of the placental enzyme 11-beta-hydroxysteroid dehydrogenase-type 2 (11 β -HSD2) (Brown et al., 1993, Brown et al., 1996b, Krozowski et al., 1995) which catalyses the conversion of active cortisol into inactive cortisone. In human placenta 11 β -HSD2 is localized to the syncytiotrophoblast (Krozowski et al., 1995), which is the primary barrier between the mother and the fetus and thus prevents glucocorticoids accessing placental cells and the fetal compartment (Chapman et al., 2013). Indeed placental 11 β -HSD2 has been suggested to inactivate the majority of maternal glucocorticoids passing to the fetus in rodents (Cottrell et al., 2012) and in humans (Benediktsson et al., 1997). 11-beta-hydroxysteroid dehydrogenase-type 1 (11 β -HSD1), which regenerates cortisol from inactive cortisone, is undetectable in the syncytiotrophoblast, but is localized in the extravillous trophoblasts (situated near maternal circulation) and endothelial cells lining fetal capillaries in terminal villi (Sun et al., 1997). Whether or not the activity of placental 11 β -HSD1 regenerates a substantial amount of cortisol or contributes significantly to maternal or fetal circulations is not well understood. With a number of studies demonstrating links between placental glucocorticoid transfer, sensitivity and metabolism and adverse outcomes in

infancy, childhood and adolescence (Reynolds et al., 2015, Raikkonen et al., 2015), understanding of the regulatory mechanisms and rate-limiting steps of maternal-fetal cortisol transfer is essential in order to identify whether there are any options for targeted intervention to improve pregnancy outcomes.

Studies using the *ex vivo* dual perfused placental perfusion model together with computational modeling have generated new mechanistic insights into placental amino acid and lipid transfer from mother to fetus (Sengers et al., 2010, Panitchob et al., 2015, Perazzolo et al., 2017). In the current study we used this combined experimental and computational modeling approach to develop a model to explore placental cortisol metabolism and transfer and its regulation. We hypothesised that activity of placental 11 β -HSD2 would be the major rate limiting step in maternal cortisol transfer to the fetus.

6.3 Methods

Term placentas from women with uncomplicated pregnancies were collected on ice immediately after delivery by elective Caesarean section at the Royal Infirmary of Edinburgh, with ethical approval (REC09/S0704/3) and written informed consent. Elective Caesarean sections were performed between 39-40 weeks of gestation. Eight placentas were collected, and experimental data was obtained from five of these. Three of these did not meet the required 'volume conservation' parameter (fetal outflow >95% of fetal inflow) during the first 30 minutes; this parameter was required for monitoring the system and estimating viability (described in section 2.4.1). These experiments were discontinued.

6.3.1 Placental Perfusions

Placentas were perfused using the methodology of Schneider (Schneider et al., 1972) as adapted in a previous study. (Cleal et al., 2007) Non-recirculating maternal and fetal circulations were established in an isolated cotyledon within

30 minutes of delivery. The fetal circulation and maternal intervillous space were perfused with a modified Earle's bicarbonate buffer (EBB: 5 mmol L⁻¹ glucose, 1.8 mmol L⁻¹ CaCl₂, 0.4 mmol L⁻¹ MgSO₄, 116.4 mmol L⁻¹ NaCl, 5.4 mmol L⁻¹ KCl, 26.2 mmol L⁻¹ NaHCO₃, 0.9 mmol L⁻¹ NaH₂PO₄), with Heparin (25,000 units/L; Fannin, Northamptonshire, UK) and bovine serum albumin (BSA [Fraction V; 98 %], 2 g/L, Sigma, UK) added. Maternal perfusate was equilibrated with 95% air and 5% CO₂, and fetal perfusate with 95% N₂ and 5% CO₂ (BOC, UK). Maternal circulation was at 14 mL/min and fetal circulation at 6 mL/min using a peristaltic pump (Watson-Marlow, UK).

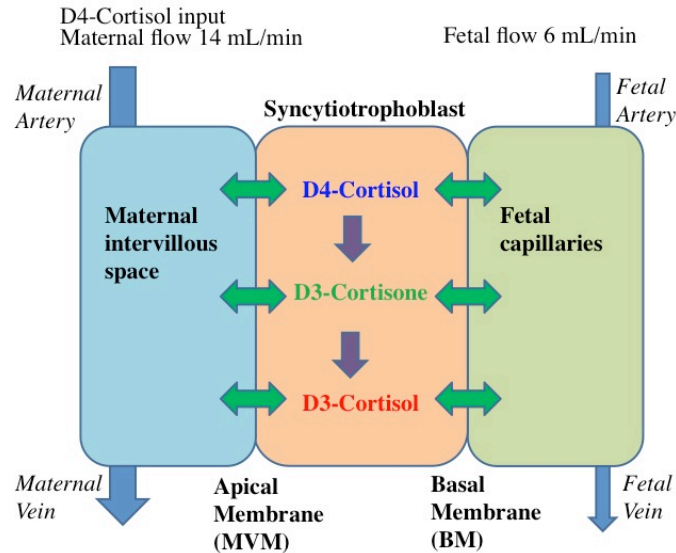
Approximately 2 mL of venous perfusate was collected from the maternal and fetal venous outflows, at 5-minute intervals. Fetal artery pressure was maintained between 40 – 70 mmHg and fetal venous return was > 95%. At the end of the experiments, the perfused mass was identified on the 'maternal side' by slight blanching. The perfused placental cotyledon was weighed. Cotyledon volume was calculated on the basis of 1 mL per g tissue. Samples of maternal and fetal perfusate fluid, un-perfused tissue and perfused tissue were stored at -80 °C until analysis.

6.3.2 Use of Deuterated Tracers to Investigate Cortisol Metabolism

Cortisol metabolism by 11 β -HSD enzymes and transport between the maternal and fetal circulations was investigated using the stable isotope deuterium (D)-labelled tracer, [9,11,12,12 ²H₄]-cortisol "D4-cortisol" (Andrew et al., 2002) which is converted to [9,12,12 ²H₃]-cortisone "D3-cortisone" by 11 β -HSD2. Measurement of [9,12,12 ²H₃]-cortisol "D3-cortisol", which is regenerated from D3-cortisone can be used to assess activity of 11 β -HSD1 (**Figure 6-1**). After an initial 'washout' period of 30 minutes, D4-cortisol (Steraloids, USA) was perfused into the maternal circulation with stepped increases in concentrations of 20 nM, 200 nM and 800 nM every 30 minutes. The 800 nM D4-cortisol concentration was considered to be representative of circulating maternal cortisol levels in the third trimester (Stirrat et al., 2016). The HSD inhibitor carbenoxolone (Sigma, UK) was added to the perfusion solution in addition to

800 nM D4-cortisol in the final 30 minutes at a concentration of 1000 nM, as informed by a previous study (Benediktsson et al., 1997).

a)



b)

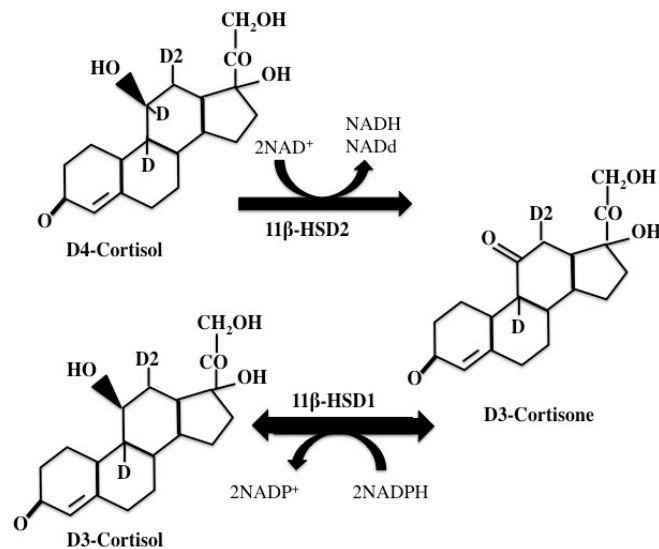


Figure 6-1 a-b. Model Schematic and Metabolism of deuterium-labelled glucocorticoids

Model schematic showing the three compartments (maternal, syncytiotrophoblast and fetal; 6-1a) distinguished in the model. It is assumed that transfer between compartments is by simple diffusion, while metabolic conversion between cortisol and cortisone takes place in the syncytiotrophoblast (Equations. 1-6, see methods section). The input concentration of D4-cortisol in the maternal compartment varies over time according to the experimental protocol, while the input concentration in the fetal compartment is zero at all times. The output concentrations of the maternal and fetal compartments from the model can be compared to the experimental data.

D4-Cortisol is inactivated by 11 β -HSD2 to D3-cortisone, with the loss of the deuterium on C11. 11 β -HSD1 regenerates D3-cortisol from D3-cortisone, with the addition of an unlabeled hydrogen (**6-1b**).

6.3.3 Steroid Extraction and LC-MS/MS Quantification

Endogenous (cortisol, cortisone) and deuterated (D4-cortisol, D3-cortisone and D3-cortisol) glucocorticoids were measured simultaneously by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters Acquity™ UPLC (Manchester, UK) liquid chromatography system followed by mass spectral detection on an ABSciex QTRAP® 5500 (Warrington, UK) operated in positive electrospray ionization mode. Mass spectral conditions are described in **Appendix Table 6-1** in conjunction with ion spray voltage (5500 V) and source temperature (700 °C).

6.3.4 Perfusate Fluid Extraction

Following enrichment of perfusate (500 μ L) with the internal standard epi-cortisol (10 ng; Steraloids, USA) and dilution with water (500 μ L) analytes were extracted using a Sep-Pak C18 40 mg 96-well plate (Waters, Manchester,

UK). Plates were primed with methanol (1 mL), then EBB (1 mL) then samples (500 μ L) were loaded and plates washed with water (1 mL). Analytes were eluted from the plate using acetonitrile (1 mL) directly into a 2 mL deep well collection plate (Waters, UK). Eluants were dried under oxygen-free nitrogen (60 °C) using a 96-well Dry down apparatus, and reconstituted in mobile phase (30:70 methanol: water; 100 μ L).

6.3.5 Tissue Extraction

Placental tissue (200 mg) was homogenized in 3 mL 7:2 methanol: water and enriched with internal standard epi-cortisol (10 ng, as above) before being centrifuged at 3200 g for 45 minutes at 4 °C. Supernatant was transferred to a clean glass vial and dried under oxygen-free nitrogen (60 °C) and reconstituted in water (5 mL). Analytes were extracted using Sep-Pak C18 360 mg Classic Cartridges (Waters). Cartridges were primed with 100% methanol (5 mL) followed by water (5 mL). Samples were added to cartridges and allowed to flow through with gravity. Cartridges were washed with water (5 mL), and analytes were eluted with 100% methanol (2 mL) into a 3.5 mL glass vial. Eluants were dried down under oxygen-free nitrogen (60 °C) and reconstituted in 100 μ L mobile phase.

6.3.6 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Samples in the auto-sampler were maintained at 10 °C. Analytes were separated at 40 °C on an ACE Excel C18-AR column (100 x 2.1 mm, 1.7 μ m; Hichrom Limited®, Berkshire, UK) at a flow rate of 0.5 mL/min. Samples in the auto-sampler and sample manager were maintained at 10 °C. Starting with 70% water with 0.1% formic acid (FA) (solution A) and 30% acetonitrile with 0.1% FA (solution B), maintained for 4 minutes followed by a 1-minute linear rise to 60% solution B, a subsequent rise to 90% solution B, before restoring to 30% solution B at 6.1 minutes. This condition was sustained for 1-minute to re-equilibrate. Inter- and intra-assay precision and accuracy are displayed in

Appendix Table 6-2.

The peak areas of deuterated steroids were corrected for the abundances of naturally occurring isotopomers at baseline. In addition, the peak area of D4-cortisol was corrected for interference from the M+4 isotopologue of cortisol and the M+1 isotopologue of D3-cortisol. The peak area of D3-cortisol was corrected for interference from the M+3 isotopologue of cortisol.

6.3.7 Data Analysis

Deuterated hormone levels were adjusted for flow rate and were normalised to tissue weight of the perfused cotyledon. D4-cortisol and D3-cortisol were reported in ng, and in the absence of a standard for accurate quantification, D3-cortisone was measured in arbitrary units.

6.3.8 Computational Model for Placental Transfer

A compartmental modeling framework was adopted to model the placental transfer of cortisol and cortisone in the *ex-vivo* perfusion experiments, based on our previous work. (Sengers et al., 2010, Panitchob et al., 2015, Panitchob et al., 2016) The model distinguishes three separate physiological compartments associated with the maternal, syncytiotrophoblast and fetal capillary volumes (**Figure 6-1a**). Each compartment is described as well mixed. Transfer between compartments is determined by the fluxes across the apical and basal membranes and assumed to occur by simple diffusion for both cortisol and cortisone. Metabolic conversion from cortisol to cortisone within the syncytiotrophoblast is described as unidirectional using Michaelis-Menten kinetics. Model equations were implemented in Matlab (R2016a) as outlined previously (Panitchob et al., 2015, Panitchob et al., 2016, Sengers et al., 2010) and resulted in the following system of equations:

$$\frac{dC_A^m}{dt} = \frac{1}{v_m} \left(Q_m (C_A^{in,m} - C_A^m) - k_{MVM} (C_A^m - C_A^s) \right) \quad [1]$$

$$\frac{dC_A^s}{dt} = \frac{1}{v_s} \left(k_{MVM} (C_A^m - C_A^s) - k_{BM} (C_A^s - C_A^f) + J_A^{metab} \right) \quad [2]$$

$$\frac{dC_A^f}{dt} = \frac{1}{v_f} \left(k_{BM} (C_A^s - C_A^f) - Q_f C_A^f \right) \quad [3]$$

where C_A^m , C_A^s and C_A^f are the concentrations (mol/L) of solute A which can be either D4-cortisol (D4F), D3-cortisone (D3E) or D3-cortisol (D3F) in the maternal “ m ”, syncytiotrophoblast “ s ” and fetal “ f ” compartment respectively. Similarly, the volumes v (L) of the different compartments are indicated with subscripts using the same notation. Q_m and Q_f (L/min) are the fluid flow rates in the maternal and fetal circulation. $C_A^{in,m}$ is the maternal inlet concentration, which is zero for all solute species except D4-cortisol. Note that the fetal inlet concentration is zero for all species and therefore has not been included. k_{MVM} and k_{BM} denote the effective overall permeability constants (L/min) for the microvillous membrane (MVM) and basal membrane (BM) including surface area. These diffusive permeability constants were assumed to be the same for all solute species. The metabolic conversion rate J_A^{metab} (mol/min) depends on the solute species as follows:

$$J_{D4F}^{metab} = -\frac{V_{4F \rightarrow 3E}^{max} C_{D4F}^s}{K_m + C_{D4F}^s} \quad [4]$$

$$J_{D3E}^{metab} = \frac{V_{4F \rightarrow 3E}^{max} C_{D4F}^s}{K_m + C_{D4F}^s} - \frac{V_{3E \rightarrow 3F}^{max} C_{D3E}^s}{K_m + C_{D3E}^s} \quad [5]$$

$$J_{D3F}^{metab} = \frac{V_{3E \rightarrow 3F}^{max} C_{D3E}^S}{K_m + C_{D3E}^S} \quad [6]$$

where V^{max} (mol/min) is the maximum overall metabolic conversion rate and K_m (mol/L) is the Michaelis-Menten constant, i.e. the concentration at which half the maximum rate occurs.

6.3.8.1 Model Parameters

The total cotyledon volume was based on the average cotyledon weight from the experiments (30.8×10^{-3} kg, $n = 5$), which was directly equated to the volume in L. The volume fractions of the maternal, syncytiotrophoblast and fetal compartments distinguished in the model were set to 34%, 15% and 7.4% respectively, as in our previous work. (Sengers et al., 2010, Mayhew, 2009) The flow rates in the maternal and fetal circulations $Q_m = 14 \times 10^{-3}$ L/min and $Q_f = 6 \times 10^{-3}$ L/min were directly based on the experimental settings. To account for any discrepancies between nominal and actual values, the D4-cortisol input concentrations C_A^{in} used in the model were calculated based on the combined maternal and fetal steady state output during the blocking phase. The Michaelis-Menten constant K_m was set to 44×10^{-9} mol/L, based on the value for the enzyme 11 β -HSD2 for cortisol (Brown et al., 1996a). In first instance the same value was adopted for both metabolic conversion steps in Equations 4-6.

6.3.8.2 Parameter Estimation

The remaining parameters in the model were determined by fitting the experimental data. The following error criterion was defined for a certain species A and compartment j in general:

$$R_A^j = \frac{1}{(\bar{C}_A^{exp,j})^2} \sum_{i \in T} (C_{A,i}^j - C_{A,i}^{exp,j})^2 \quad [7]$$

where $C_{A,i}^j$ and $C_{A,i}^{exp,j}$ are the computed and experimental concentrations at time point i , respectively, while $\bar{C}_A^{exp,j}$ is the mean of the experimental time points considered. The model was fitted to the steady state values after each change in maternal input concentration, including the blocking phase, therefore the set of time points T consisted of the last 4 time points for each different input phase (16 time points in total).

The D3-Cortisol concentrations measured experimentally were 300 times smaller compared to D4-Cortisol and did not contribute significantly to the overall mass balance. Therefore the conversion to D3-Cortisol was neglected in the parameter estimation by setting $V_{3E \rightarrow 3F}^{max}$ to zero. In addition, the measured D3-Cortisone values could not be directly related to concentration. Therefore D3-Cortisone was not fitted, but instead the experimental values for D3-Cortisone were scaled to allow comparison of the relative changes predicted by the model. Thus, only the D4-Cortisol values in the maternal and fetal compartments (averaged over 5 placentas) were fitted according to the following overall error criterion:

$$R_{tot} = R_{D4F}^m + R_{D4F}^f \quad [8]$$

In total 3 parameters were fitted, the membrane permeability constants k_{MVM} and k_{BM} and the maximum rate of conversion from cortisol to cortisone $V_{4F \rightarrow 3E}^{max}$. Time integration of Equations 1-3 was performed in Matlab (R2016a) using the *ode45* function (Runge-Kutta (4, 5) method). Parameter estimation by minimising Eq. 8 was implemented using the *fminsearch* function (Nelder-Mead method). Initial parameter estimates were varied to verify that the algorithm converged to a unique solution.

A sensitivity analysis was carried out in which the model parameters were varied with respect to the values for the reference fit. The reported changes in placental transfer predicted by the model were based on the steady state results at the highest maternal input concentration.

6.4 Results

6.4.1 Subject Characteristics

The mean (sd) maternal age was 36.4 ± 6.3 years, mean gestational length was 277 ± 2 days ($39+4$ weeks ± 2 days), and mean birthweight was 3721 ± 223 g.

6.4.2 D4-cortisol, D3-cortisone and D3-cortisol levels

Figure 6-2 shows the levels of D4-cortisol, D3-cortisone and D3-cortisol (plotted data with error bars) in maternal and fetal veins increased as the concentration of D4-cortisol in the maternal artery perfusion increased. D4-cortisol (**Figure 6-2 a-b**) and D3-cortisone (**Figure 6-2 c-d**) were detected in maternal and fetal vein 5-minutes after commencement of D4-cortisol perfusion (20 nM) in the maternal artery. D3-cortisol (**Figure 6-2 e-f**) at 95-minutes into the experiment in the maternal vein (perfusion phase: 800 nM D4-cortisol), and at 75-minutes in the fetal vein (perfusion phase: 200 nM D4-cortisol). The biggest increase in D4-cortisol and D3-cortisone levels occurred when maternal artery D4-cortisol perfusion increased from 200 nM to 800 nM. Levels of D3-cortisone in the maternal circulation were approximately 5-fold higher than in the fetal circulation. When carbenoxolone was added to the maternal artery perfusion, D4-cortisol levels further increased in maternal and fetal veins, and D3-cortisone synthesis was completely inhibited. D3-cortisol levels were around 300-fold lower than D4-cortisol in both maternal and fetal circulations, and were close to the assay limit of detection. Levels of D3-cortisol in the maternal circulation were approximately 2-3-fold higher than levels in the fetal circulation. Proportionately more of the produced D3-cortisol was released into

the fetal circulation than maternal circulation, when compared with the proportion of D3-cortisol released into maternal and fetal circulations. Samples of buffer obtained on completion of the ‘washout’ phase of the experiment confirmed that there were no remaining endogenous or labelled glucocorticoids within the tubing used for the circuit.

6.4.3 Placental Model Results

The results of the model fit of the average maternal and fetal D4-cortisol measurements demonstrated an excellent overall ability of the computational model to represent the experimental data (**Figure 6-2, Appendix Table 6-3**). From the model the estimated effective membrane permeability constant $k_{MVM} = 0.011$ L/min for the maternal facing MVM and $k_{BM} = 0.0015$ L/min for the fetal facing BM. Thus the permeability of the MVM was estimated to be 7.4 times higher than that of the BM. The estimated maximum rate capacity for the conversion of cortisol into cortisone $V_{4F \rightarrow 3E}^{max} = 5.0$ nmol/min per cotyledon. At the highest inlet concentration only 3.0% of the maternal cortisol input was transferred to the fetal circulation, while 26.5% was metabolised and the remaining 70.5% exited via the maternal vein. Inhibiting 11 β -HSD activity increased the transfer to the fetus to 7.3% of the maternal input, while 92.7% exited via the maternal vein. Based on these results it can also be seen that enzyme metabolism reduced transfer to the fetus by 59%. Note that if there were no placental barrier and no metabolism then the maternal and fetal vein would have an output of respectively 70% and 30% of the maternal inlet, based on the difference in flow rates alone (i.e. if concentrations within the placenta were perfectly mixed). The comparison between the predicted D3-cortisone and the scaled experimental data is shown in **Figure 6-2**. It can be observed that the relative steady state levels correspond well for the fetal D3-cortisone, while the maternal D3-cortisone shows some larger discrepancies. In addition, the model responds much more rapidly to changes in input conditions. In this respect, the sharp peak at $t = 150$ min predicted by the model is due to the absence of blocker in the washout buffer, which is assumed to take immediate effect in the model.

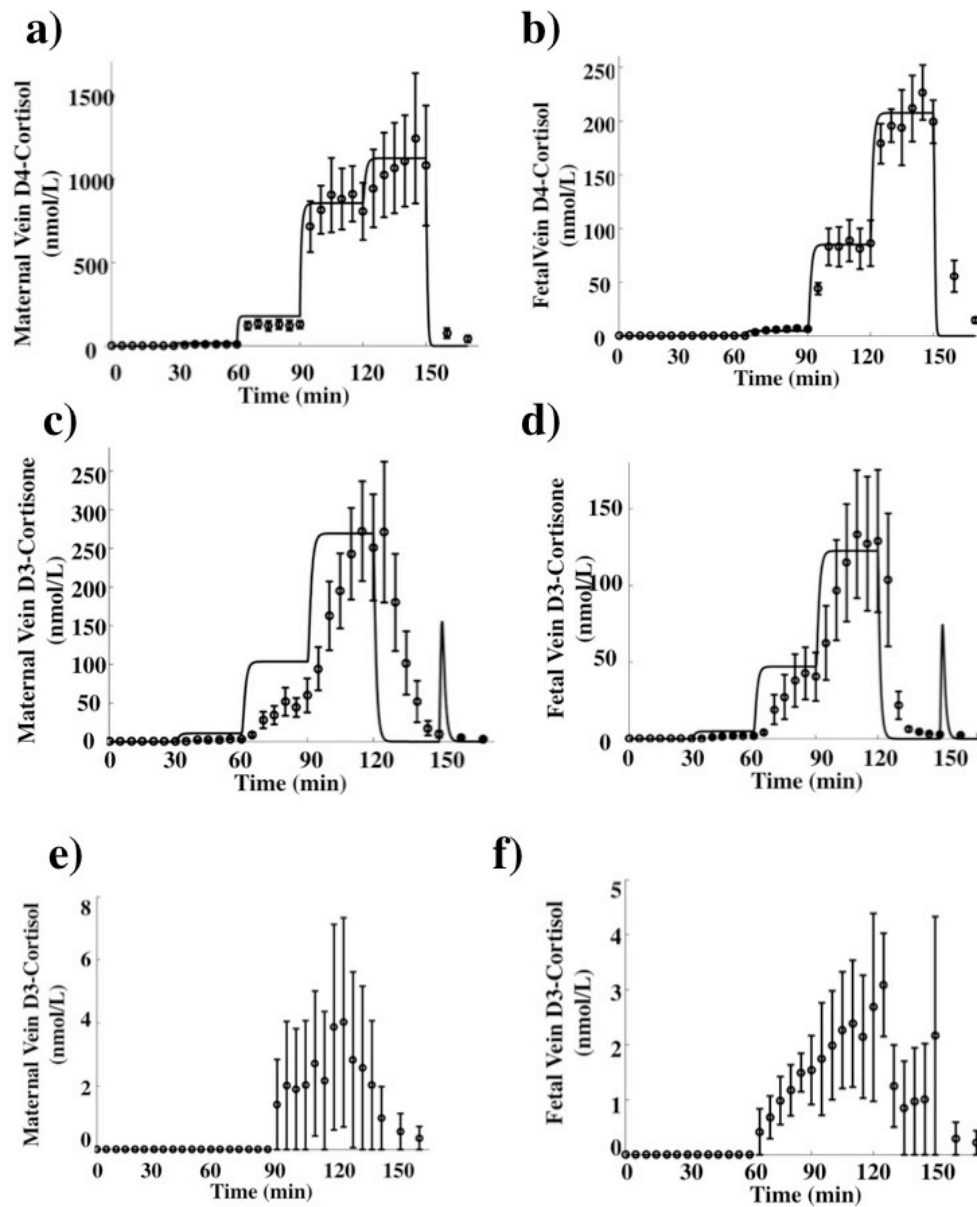


Figure 6-2 a-f. Model fit of experimental data

In maternal circulation was 0-30 minutes EBB alone, 30-60 minutes EBB + 20nM D4-Cortisol, 60-90 minutes EBB + 200nM D4-Cortisol, 90-120 minutes EBB + 800nM D4-Cortisol, 120-150 minutes EBB + 800nM D4-Cortisol + 0.001M Carbenoxolone, 150-170 minutes EBB alone. The appearance of D4-cortisol in the fetal circulation is consistent with free transplacental passage of

D4-cortisol. Inactivation of D4-cortisol by 11 β -HSD2 is indicated by the appearance of D3-cortisone in the maternal or fetal circulations, and cortisol regeneration from D3-cortisone is indicated by the appearance of D3-cortisol.

Model fit of the experimental data for D4-cortisol in the maternal (**Figure 6-2a**) and fetal (**Figure 6-2b**) compartments, with a single set of parameters. Results show an excellent correspondence between model (straight line) and experiments (plotted data and error bars) ($R^2 = 0.99$). Model prediction of D3-cortisone in comparison with the scaled experimental data (**Figure 6-2 c-d**). Note the experimental units for D3-cortisone could not be directly related to concentration and have been scaled here to allow comparison of the relative changes predicted by the model. The scale factor was derived by scaling the experimental values to the model predictions for the highest input concentration. A single scale factor was determined by averaging the ratio of the model predictions and the steady state values of the maternal and fetal concentrations. The same conversion factor was applied to both maternal and fetal D3-cortisone based on the average ratio between experimental units and computed concentrations at the highest input level (time points $t = 110, 115$ and 120 min). Experimental data for D3-cortisol (**Figure 6-2 e-f**). Values were comparatively low and were not modeled as they do not contribute significantly to the overall mass balance. All experimental results are the average of 5 placentas, expressed as mean and SEM ($n = 5$).

Key: D4F (D4-Cortisol), EBB (Earle's Bicarbonate Buffer), CBX (carbenoxolone).

The results of the sensitivity analysis in **Figure 6-3** show that when varying single parameters the placental transfer of cortisol was affected most by changes in k_{BM} , the membrane permeability of the BM, and the metabolic conversion rate of cortisol into cortisone $V_{4F \rightarrow 3E}^{max}$. In addition, placental transfer

was predicted to be moderately sensitive to k_{MVM} , the permeability of the MVM, and the maternal flow rate used in the experiment Q_m . Variations in K_m only had a small impact as the metabolism continued to operate in the saturated regime, while increasing the fetal flow rate Q_f used in the experiment was predicted to only have a minor effect on transfer. Steady state transfer was not sensitive to any of the compartment volumes, as expected. To evaluate the impact of the overall membrane permeability, an additional study was done in which k_{BM} and k_{MVM} were both varied simultaneously, demonstrating a considerably larger effect than for the permeability of each membrane separately (**Figure 6-3**).

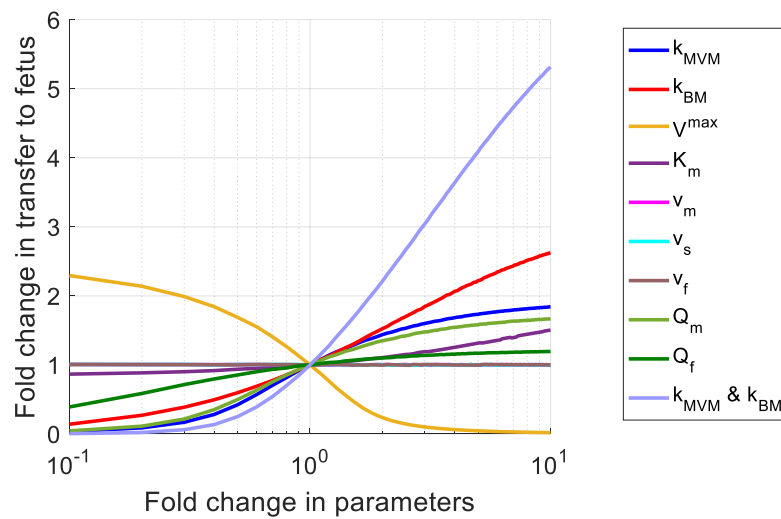


Figure 6-3. Sensitivity analysis for D4-Cortisol transfer to the fetus as a function of variations in the model parameters

The model parameters were varied with respect to the values for the reference fit. The reported changes in placental transfer predicted by the model were based on the steady state results at the highest maternal input concentration.

Key: k_{MVM} (MVM permeability constant), k_{BM} (BM permeability constant), V^{max} (maximum rate of reaction), K_m (Michaelis-Menton constant), V_m (maternal compartment volume), V_s (syncytiotrophoblast compartment volume), V_f (fetal compartment volume), Q_m (maternal flow rate, L/min), Q_f (fetal flow rate, L/min).

6.5 Discussion

The experiments performed in this study using a deuterated cortisol tracer in the *ex vivo* placental perfusion model allowed investigation of the role of interconversion of cortisol and its inactive metabolite cortisone on transfer of cortisol from mother to fetus. The application of computational modeling enabled interpretation of the transfer mechanisms that underlie these processes. Our findings challenge the concept that maternal cortisol diffuses freely across the placenta, confirm that 11 β -HSD2 acts as a major ‘barrier’ to cortisol transfer to the fetus and show preliminary evidence of local cortisol regeneration within the placenta.

Addition of carbenoxolone (a potent HSD inhibitor) to the maternal artery perfusion, resulted in no further production of D3-cortisone. This supports the role of 11 β -HSD2 as a key player in the maternal barrier to fetal glucocorticoid exposure. The activity (but not mRNA) of 11 β -HSD2 has been shown to decrease in the last two weeks before parturition (Murphy and Clifton, 2003). The placentas used in the experiments were obtained from elective Caesarean sections at between 39-40 weeks gestation, so it is not known when parturition would have occurred in these pregnancies. The model allowed an estimation of the maximum capacity of 11 β -HSD2 for conversion of cortisol to cortisone as 5.0 nmol/min per cotyledon. It is not known what the capacity of 11 β -HSD2 would be if exposed to high levels of maternal glucocorticoids for more prolonged periods, but studies have demonstrated that 11 β -HSD2 mRNA and activity is down-regulated by maternal stress (O'Donnell et al., 2012) and

inflammatory diseases (Murphy and Clifton, 2003). Further, inhibition of 11 β -HSD2 by maternal liquorice consumption has adverse consequences on child development (Raikkonen et al., 2009, Raikkonen et al., 2017). Our study supports the premise that the adverse offspring outcomes are due to increased fetal glucocorticoid exposure as when 11 β -HSD2 was inhibited by carbenoxolone, transplacental passage of maternal cortisol to the fetal circulation was more than doubled.

Yet, even when 11 β -HSD activity was inhibited using carbenoxolone, less than 10% of maternal D4-cortisol crossed the placenta in our experiments. This observation challenges the concept that cortisol freely diffuses across the placenta, and suggests alternate mechanisms to protect the fetus from high maternal cortisol levels in addition to the well described inactivation of cortisol by 11 β -HSD2. Three ABC-transporters; multidrug-resistant protein (MRP1, encoded by *ABCC1*), p-glycoprotein (P-gp, encoded by *ABCB1*) and breast-cancer-resistant protein (BCRP, encoded by *ABCG2*) are localised to placental syncytiotrophoblast, and the fetal vessel endothelium (St-Pierre et al., 2000, Yeboah et al., 2006) consistent with the potential for active transport of cortisol in and out of the placenta. Further studies are needed to investigate the contribution of ABC transporters, levels of which are known to alter across gestation (Iqbal et al., 2012, Kalabis et al., 2005, Pascolo et al., 2003, Sun et al., 2006), in regulating maternal cortisol transfer to the fetus and in particular to understand the kinetics of efflux transporters, which our preliminary observations suggest may also protect the fetus.

Further we observed approximately a 5-fold higher D3-cortisone release to the maternal circulation compared with the fetal circulation. It also needs to be considered that the physical process of cortisol diffusion across tissues may be more challenging than has been thought previously. In particular, in the placenta diffusion across the water filled villous stroma may prove a barrier to cortisol diffusion. This is consistent with the observation that cortisone was preferentially released into the maternal circulation (2:1 maternal:fetal circulation), and the lower placental to fetal permeability calculated within the model.

A novel finding is the observation of regeneration of cortisol, as evidenced by the detection of D3-cortisol in both maternal and fetal circulations. Though the absolute levels of D3-cortisol were low, this regeneration of cortisol may have local paracrine roles and increased placental 11 β -HSD1 mRNA levels have been associated with maternal depression and with altered infant regulatory behaviours (Raikkonen et al., 2015, Reynolds et al., 2015). Further, proportionately more D3-cortisol was transferred to the fetus than D3-cortisone, which is in line with localisation of 11 β -HSD1 to the endothelium (Sun et al., 1997). The computational model provided a good overall representation of the experimental data under different experimental conditions. In general, the compartmental model showed a faster response due to the well-mixed assumption, but this did not affect the steady state levels. The model predicted that changing membrane permeability of the BM would affect placental transfer of cortisol. Placental transfer of lipids has been reported to be increased in pre-eclampsia (Huang et al., 2013). Further studies are required to investigate whether inflammatory conditions such as pre-eclampsia and preterm labour alter the permeability of the BM, and thus alter placental cortisol transfer.

Our study has several limitations. Our experiments were conducted using EBB buffer and albumin. The findings may be altered *in vivo* with the presence of corticosteroid binding globulin (CBG), the primary binding protein for cortisol (Hammond, 2016) and this should be considered in future studies. Including such binding effects would not affect the overall modeling results if the unbound fraction is constant in the concentration range used, but would become important if binding differs between compartments. We were also unable to accurately quantify D3-cortisone concentrations, as there are no available standards. Nevertheless, we were able to estimate fold-changes in D3-cortisone concentrations so this should not limit interpretation of the results. A caveat of the model is that it does not account for further interconversion of D3-cortisol to D3-cortisone, although the net values of D3-cortisol quantified were very low. We did not study other pathways of cortisol metabolism such as the A-ring reductase enzymes, although Benediktsson et al., 1997 found that the products of 5 β -reductase or 20 α / β -hydroxysteroid dehydrogenase did not co-elute with

cortisol or cortisone in placental perfusion studies, suggesting that these pathways may not metabolise cortisol or cortisone in the placenta. The contribution of other potential metabolism pathway, such as via carbonyl reductase 1 (Phillips et al., 2014) which is located in placenta, is also unknown. Direct measurement of arterial input concentrations would also have provided additional confidence to this analysis.

The success rate of placental perfusions is around 50% (Myllynen and Vahakangas, 2013) although reasons for this are not well understood. In this study five out of eight placentas were successfully perfused (62.5%). Unsuccessful perfusions were discontinued due to volume loss (leak) from the maternal to fetal side. The ‘failure rate’ of experiments could be considered a limitation of this study by eliminating some placentas, reasons for volume loss are not well understood, and a recent study showed that volume loss was not affected by placental weight, way of delivery or gestation at delivery (Karttunen et al., 2015). The success of select placentas could also be considered as a strength of this study, with a relatively low variance in the successful five placentas.

Further studies using this model could investigate in more detail the contribution of the fetal circulation to maternal cortisol levels. Regeneration of cortisol from cortisone could be studied by perfusing the fetal circuit with D2-cortisone (Hughes et al., 2012), and measuring the regenerated cortisol in the maternal or fetal circuits. The potential for free placental passage of cortisol from the fetal to maternal circuit could be studied by perfusing the fetal circuit with D4-Cortisol and measuring D4-Cortisol, D3-Cortisone and D3-Cortisol in the maternal circulation. Future studies utilising inhibitors of ABC transporters are also needed to assess their contribution to placental cortisol transport.

To conclude, we have developed a model to predict maternal-fetal cortisol transfer, which can now be used in future experimental design. Further studies are now needed to refine and develop the model in order to improve understanding of the mechanisms underlying maternal-fetal cortisol transfer and the pathways to normal fetal growth.

6.6 Appendices

	Molecular Weight (amu)	Precursor ion (m/z)	Product ion (m/z) Quan; Qual	Declustering Potential (V)	Collision energy (V) Quan; Qual	Cell exit potential (V) Quan; Qual
ANALYTES						
D4-cortisol	367.0	367.0	121; only one	121	25	20
D3-cortisol	366.0	366.0	121.1; only one	121	25	20
D3-cortisone	363.2	364.2	164.0; only one	166	31	14
INTERNAL STANDARDS						
Epi-cortisol	363.2	363.2	121.0; 77.0	131	29; 101	14; 14

Appendix Table 6-1. Mass spectral conditions for analysis of analytes and internal standards by positive ion electrospray ionisation

Key : Atomic mass units (amu) Quan (quantifier ion), Qual (qualifier ion), V (volts)

		Concentration (ng/200 μL perfusate or mg tissue*); mean (SD)	Precision (% RSD)	Accuracy (%)
D4- Cortisol	Low (0.2)	0.21 (0.02)	10.5	103
	Mid (50)	46.7 (1.7)	3.6	93
	High (400)	396.6 (43.4)	11.6	93
	Tissue Sample*	8.3 (0.6)	7.0	
D3- Cortisol	Low (0.1)	0.1 (0.02)	17.3	98
	Mid (10)	10.4 (0.9)	8.8	104
	High (20)	20.2 (2.0)	10.1	101
	Tissue Sample*	0.6 (0.04)	6.4	

Appendix Table 6-2. Inter-assay precision and accuracy

Concentrations of cortisol, cortisone, D4-Cortisol and D3-Cortisol were determined using calibration curves. Fourteen standards were prepared in 500 μ L EBB (range of concentrations 0.1 ng – 400 ng) enriched with internal standards (10 ng) along with blank samples were diluted in 500 μ L of water and processed using the same extraction method and analysis conditions as perfusate samples. Standard curves were plotted by calculating the peak area (analyte peak area / internal standard peak area). Weighting of 1/x and was applied to form standard curves of best fit with a regression coefficient above 0.99. The ion ratio (quantitative ion/qualitative ion) of the analytes was calculated using MultiQuant software and results were not considered acceptable if the ratio was greater than 20% of the ratio of the standards. Inter-assay fourteen point standard curve validation (n=6 different day respectively)

was used to assess the limits of quantification of accuracy and precision for each analyte. Precision was based on the percentage relative standard deviation (%RSD), which was calculated using peak area ratios. Tissue sample* is intra-assay (amount, ng for tissue replicates (n=6). Inter-assay was not performed for tissue samples, as all tissue samples were analysed on the same day. Low values are the limit of quantification for each analyte.

Key: EBB (Earle's Bicarbonate Buffer), SD (Standard Deviation), RSD (Relative Standard Deviation)

		Maternal Perfusion Concentration			
		20nM D4-Cortisol	200nM D4-Cortisol	800nM D4-Cortisol	800nM D4-Cortisol + CBX
Measured Values	Maternal vein D4-cortisol nmol/L D3-cortisol nmol/L	11.5 (5.1) 0	127.7 (51.2) 0	806.9 (382.3) 3.6 (6.3)	1243 (779.8) 1.3 (4.7)
	Fetal Vein D4-cortisol nmol/L D3-cortisol nmol/L	0.18 (0.2) 0	6.7 (1.4) 1.5 (1.4)	86.4 (47.7) 2.7 (3.8)	199.4 (40.0) 2.9 (5.0)
	Model Values				
	Maternal artery D4-cortisol nmol/L	30.3	303.4	1213.5	1213.5
	Maternal vein D4-cortisol nmol/L D3-cortisone nmol/L	17.4 10.7	177.6 103.5	855.2 599.4	1124.5 0
	Syncytiotrophoblast D4-cortisol nmol/L D3-cortisone nmol/L	1.6 23.75	23.5 230.4	416.1 599.4	1015.5 0
	Fetal vein D4-cortisol nmol/L D3-cortisone nmol/L	0.334 4.85	4.79 47.1	85.04 122.5	207.58 122.5
	D4-cortisol Transfer	0.47%	0.68%	3.0%	7.3%

Appendix Table 6-3. Deuterated glucocorticoid concentrations in the maternal, syncytiotrophoblast and fetal compartments

Glucocorticoid concentrations quickly reached a new constant equilibrium for each input concentration and steady state values were taken at the end of each perfusion phase, just before the input concentrations changed. ‘Measured values’ are mean (sd) quantified from LC-MS/MS data from five placental perfusion experiments, and adjusted for flow rates. ‘Model values’ were derived from the computational model. Maternal artery concentrations were derived from the outputs during the carbenoxolone inhibition phase at the highest input level (800nM cortisol), and then decreased proportionally for the 200nM and 20nM input phases. ‘D4-cortisol Transfer’

from the maternal to fetal compartment was calculated as a percentage of D4-cortisol in the fetal vein, from the maternal artery D4-cortisol input.

Key: Carbenoxolone (CBX)

6.7 Chapter Conclusion

The placental perfusion studies in **Chapter 6** challenge the belief that maternal cortisol freely diffuses across the placenta, and confirm that 11 β -HSD2 acts as a major ‘barrier’ to cortisol transfer from the mother to the fetus. Computational modeling suggested that at the highest concentration of maternal cortisol perfusion (with deuterated cortisol), only 3% of maternal cortisol input was transferred to the fetal circulation, while 26.5% of maternal cortisol was metabolised and the 70.5% remained in the maternal circulation. Inhibiting the activity of the 11 β -HSD enzymes resulted in only a small increased in the proportion of maternal cortisol transferred to the fetal circulation.

The model described in **Chapter 6** could be used in further studies to examine the effect of maternal BMI on placental cortisol transfer and metabolism.

Chapter 7

Discussion

7.1 General Discussion / Summary of Results

The work presented in this Thesis was based on the hypothesis that the HPA axis activity is dysregulated in maternal obesity, and that this may be associated with altered release, clearance and placental cortisol metabolism, and influence clinical outcomes related to fetal size and length of gestation. This was carried out by studying the relationship between maternal obesity, a number of measures of the HPA axis, associations within clinical outcomes, and investigating possible underlying mechanisms.

The prevalence of obesity and therefore maternal obesity is increasing. As maternal obesity increases, the risks of the sequelae for offspring of obese who were fetuses in the obesogenic environment also increases. This may be a self-perpetuating cyclical problem. With this increase in mind, and the links made to offspring health outcomes later in life, it is becoming increasingly important to be able to identify the mechanisms by which maternal obesity affects the mother and the baby, in the short term and in the long term. It will be important to identify therapeutic targets and agents, as well as biomarkers for disease risk in the increasing population at risk.

This Thesis showed that there are significant effects of maternal obesity on the HPA axis hormones. Glucocorticoids are important for fetal-placental growth and maturation, and excess fetal exposure to glucocorticoids has been implicated in fetal programming, therefore this observation is of great importance.

In **Chapter 3**, it was shown that the activity of the HPA axis is decreased in maternal obesity, and that this associates with increased fetal size and longer gestation. Maternal cortisol, CRH, estrogens and progesterone were lower in obese pregnancy. Total urinary glucocorticoid metabolites increased significantly in lean pregnancy, but not in obese, suggesting that the lower levels of circulating cortisol in obese were not due to increased urinary clearance. Lower maternal cortisol in obese tended to be associated with increased birthweight. In obese, CRH at 28 weeks correlated inversely with gestational length, and independently predicted gestational length after adjustment for confounding factors.

In **Chapter 4**, it was shown that active glucocorticoid hormones (cortisol and corticosterone) were significantly higher in maternal than cord blood. Inactive versions (cortisone and 11-dehydrocorticosterone, respectively) were significantly lower in cord than maternal blood. Increased maternal BMI was associated with lower maternal cortisol, corticosterone and 11-dehydrocorticosterone. Despite significant positive correlations between maternal and cord blood glucocorticoid levels, increased maternal BMI was not associated with lower cord blood glucocorticoid levels. This suggests that while the pattern of lower maternal cortisol described in **Chapter 3** was maintained in maternal blood at the time of delivery, that conditions at delivery may overcome any potential negative effects of low maternal glucocorticoids on the fetus in the short-term. This may not preclude the longer-term effects of exposure of the fetus to lower glucocorticoid levels during obese pregnancy.

In **Chapter 5**, the release of glucocorticoid hormones was studied. It was shown for the first time that cortisol is pulsatile in interstitial fluid during pregnancy, and that pulse frequency decreases with advancing gestation in obese pregnancy. Serum cortisol AUC, highest peak, and lowest trough increased with advancing gestation in lean and obese pregnant compared with non-pregnant controls. These findings suggest that changes in the ultradian profile of cortisol influence overall

glucocorticoid exposure, so they are important. This may be a mechanism to explain decreased HPA activity in obese pregnancy. This may be a novel pathway to obese related pregnancy outcomes such as adverse fetal growth.

In **Chapter 6**, a deuterated cortisol tracer was used in the *ex vivo* placental perfusion model, in combination with computational modeling, to investigate the role of interconversion of cortisol and its inactive metabolite cortisone on transfer of cortisol from mother to fetus. It was shown that at the highest inlet perfusion concentration of D4-cortisol, that only 3% of maternal cortisol input was transferred to the fetal circulation, while 26.5% was metabolised and the remaining 70.5% exited via the maternal vein. Inhibiting 11 β -HSD activity increased the transfer to the fetus to 7.3% of the maternal input, while 92.7% exited via the maternal vein. D3-cortisol was detected, suggesting for the first time that the placenta regenerates cortisol from cortisone. These findings challenge the concept that maternal cortisol diffuses freely across the placenta, confirm that 11 β -HSD2 acts as a major ‘barrier’ to cortisol transfer to the fetus, and show preliminary evidence of local cortisol production within the placenta. This is in keeping with other studies suggesting that the placenta acts as a barrier to prevent the developing fetus from overexposure to cortisol during pregnancy.

7.2 Wider and Clinical Implications

7.2.1 Dysregulation of the HPA Axis in Obese Pregnancy

While it has long been recognized that obesity is associated with a dysregulated HPA axis, a recent systematic review (Incollingo Rodriguez et al., 2015) examining evidence for HPA axis dysregulation in obese individuals concluded that available evidence remains inconclusive. Evidence included multiple indices of HPA axis activity, including the cortisol awakening response, diurnal cortisol slope, total daily

output, reactivity, feedback sensitivity, and the long-term output. Inconsistencies in the data may relate to the different measures of obesity used (ie. generalized obesity or abdominal obesity), and variation in protocol. Although inconsistent, the evidence does support that obesity is linked to dysregulation of the HPA axis. It was proposed that the cumulative effect of increased cortisol reactivity, local regeneration of cortisol (Walker and Andrew, 2006) and increase clearance was postulated to contribute to lower levels of systemic cortisol (Putignano et al., 2001, Duclos et al., 2001). This Thesis is one of a very few studies in humans investigating the effects of maternal obesity on the HPA axis. It found lower fasting morning cortisol levels in very severely obese women throughout pregnancy compared with a lean control group; a pattern similar to non-pregnant severe obesity (Champaneri et al., 2013). The lack of an increase in urinary clearance of glucocorticoids during pregnancy in obese women suggests that in contrast to non-pregnant obesity (Strain et al., 1980, Marin et al., 1992), the activation of the HPA axis which normally occurs during pregnancy (Duthie and Reynolds, 2013, Lindsay and Nieman, 2005) was blunted in these very severely obese pregnant women.

7.2.2 Implications for Obese Related Pregnancy Outcomes

Dysregulation of the HPA axis may underlie some obese related adverse pregnancy outcomes. As excess glucocorticoids are associated with fetal growth restriction (Goedhart et al., 2010, Bolten et al., 2011, Kivlighan et al., 2008) and preterm labour (Sandman et al., 2006), our finding of an association between lower cortisol and CRH with increased fetal size and longer length of gestation may explain the increased rates of macrosomia (Ehrenberg et al., 2004) and prolonged pregnancy (Denison et al., 2008, Heslehurst et al., 2017) reported in obese pregnancy. These findings also further support the hypothesis that CRH acts as a ‘placental clock’, by playing a role in governing the length of gestation and triggering the onset of labour

(Sandman et al., 2006).

Glucocorticoids have been shown to down regulate placental glucose transporters (Hahn et al., 1999) and reduce lipid transport to the fetus (Magnusson-Olsson et al., 2006). CRH has been shown to reduce amino acid transport (Giovannelli et al., 2011) and decrease mRNA expression of the GLUT3 amino acid transporter in the placenta (Gao et al., 2012). Estradiol, estriol and progesterone have also been shown to inhibit transport of monosaccharides in the placenta (Johnson and Smith, 1980). The lower levels of cortisol, CRH estradiol, estriol and progesterone in obese pregnancy observed in this work could be a mechanism for increased nutrient transport to the fetus in obese pregnancy, which could be a mechanism for increased fetal growth.

7.2.3 Longer Term Offspring Outcomes

Fetal exposure to excess glucocorticoids *in utero* has been linked to the development of cardio-metabolic risk factors and disease later in life (Reynolds, 2013). Offspring of obese are also more likely to develop obesity and cardio-metabolic risk factors (Drake and Reynolds, 2010). A recent data-linkage study has suggested that these associations may translate into increased risk of cardiovascular disease and death for offspring of obese mothers (Reynolds et al., 2013).

The findings in this Thesis suggest that offspring of obese are exposed to comparatively lower levels of glucocorticoids *in utero*. The longer-term effects of this are not known, but may include cardio-metabolic risk factors and disease.

A recent follow-up study of the offspring of the cohort in **Chapter 3** found that first trimester maternal cortisol correlated with offspring salivary cortisol under basal and experimental conditions, and higher cortisol exposure in the third trimester was associated with offspring cortisol profile under basal conditions (Mina et al., 2017).

This finding is consistent with other studies that linked fetal cortisol exposure in late pregnancy to HPA responses in offspring (Yehuda et al., 2005).

Another follow-up study (Mina et al., 2016) of these offspring reported that maternal obesity was a significant predictor of increased neuropsychiatric problems, externalizing behavior problems including symptoms of attention deficit hyperactivity disorder (ADHD) and aggressive behaviour, sleep problems and neurodevelopmental problems in their children. These findings were independent of maternal gestational diabetes (GDM), and the authors suggested that potential underlying causes could include maternal chronic inflammation (Bilbo and Schwarz, 2012, Brown et al., 2014) and altered glucocorticoid exposure either directly through altered placental glucocorticoid barrier (Raikkonen et al., 2015) and / or epigenetic modification of glucocorticoid receptors (Heinrich et al., 2015, Parade et al., 2016). These studies provide further support for the hypothesis that exposure to maternal obesity and altered glucocorticoid exposure has longer-term consequences for the offspring.

7.2.4 Possible Mechanisms – Altered Ultradian Rhythm

To the best of our knowledge, the work in this Thesis is the first work to conduct dynamic tests of the ultradian rhythm in pregnancy, and in obese pregnancy. Altered pulsatility of glucocorticoid hormones may underlie the decreased HPA axis activity we observed in obese pregnancy. There has been increasing interest in the role of cortisol pulsatility in transcriptional regulation of glucocorticoid responsive genes. As disruptions in pulse characteristics have been reported in pathological states that relate to altered glucocorticoids such psychotic and depressive states (Young et al., 1994, Young et al., 2004, Deuschle et al., 1997) and Cushing's syndrome (van Aken et al., 2005, Boyar et al., 1979), it is possible that the decreased frequency of cortisol

pulses in interstitial fluid we observed in **Chapter 5** could underlie the lower levels of maternal cortisol in obese reported in **Chapters 3-4**, and that these could be implicated in obese related adverse pregnancy outcomes that may relate to glucocorticoids. Animal studies have also suggested that it is the pulsatile pattern rather than the absolute concentrations of glucocorticoids exposure that determines subsequent responsiveness to stress, and this has obvious implications for understanding the pathogenesis of stress-related disease (Sarabdjitsingh et al., 2010a, Sarabdjitsingh et al., 2010b).

There is an increasing body of evidence suggesting that fetal growth is influenced by both genetic and environmental factors, but the relevant molecular pathways are not well understood (Moore et al., 2015). Genetic factors influencing fetal growth are thought to include imprinted genes (Abu-Amero et al., 2006, Moore et al., 2015) and DNA methylation (Koukoura et al., 2012). Methylation of the GR gene exon 1F in the human placenta has been reported to be associated with large for gestational age (Filiberto et al., 2011) and increased placental 11 β -HSD2 methylation has been reported in infants with lower birthweight (Marsit et al., 2012). Methylation of 11 β -HSD2 in peripheral blood cells in adulthood has also been linked to both neonatal anthropometric variables and risk factors for cardiovascular disease in adulthood (Drake et al., 2012). Given the links between genetic factors and fetal growth, we can speculate that altered ultradian rhythm of glucocorticoids (known to associate with other disease processes) could also be another mechanism influencing fetal growth. Identifying key genes and pathways that regulate fetal growth will allow for better monitoring of intrauterine growth, maximizing healthy outcomes.

It has been suggested that more than 25% of the variation in length of gestation could be due to genetic factors (Clausson et al., 2000). Post term, prolonged pregnancy is thought to have a large heritability, which implicates genetic and or epigenetics as contributing factors on gestational age (Oberg et al., 2013, Laursen et al., 2004, Lunde et al., 2007, Kistka et al., 2007). However, no studies have found any specific

common genetic or epigenetic variants to be associated directly with prolonged gestation.

With an increasing body of evidence suggesting that genetic factors are linked to fetal growth, the length of gestation, and cardiovascular health, and reports that disruptions in glucocorticoid pulse characteristics are associated with disease processes, we can speculate that an altered ultradian rhythm of cortisol may underlie these obese related pregnancy outcomes.

7.2.5 Possible Mechanisms – Altered Placental Function

Placental function may contribute to, or be influenced by altered HPA axis activity in obese pregnancy. A better understanding of placental cortisol metabolism and transport will facilitate studies of the effect of maternal obesity on placental function.

The findings in **Chapter 4** of significantly higher levels of active glucocorticoids (cortisol and corticosterone) in maternal blood, and significantly higher levels of inactive versions (cortisone and 11-dehydrocorticosterone) in cord blood, are in-keeping with the hypothesis that the placenta acts as a barrier to cortisol transfer to the fetus. This finding was confirmed in **Chapter 6**, where the addition of carbenoxolone (11 β -HSD inhibitor) to the maternal placental perfusion circulation, resulted in no further production of D3-cortisone.

The findings in **Chapter 6** challenged the concept that cortisol can freely cross the placenta from maternal to fetal compartments, suggesting that alternative mechanisms may protect the fetus from higher maternal cortisol. Potential mechanisms may include the ABC-transporters, which as discussed in **Chapter 6**, may be altered in obese pregnancy. A recent study (Mina et al., 2015) using placentas from the pregnancies in the cohort in **Chapter 3** found significantly lower

levels of the ATP-binding cassette transport protein transporter encoded by *ABCB1* in obese, which actively pumps glucocorticoids from the fetal to the maternal circulation in rodents (Kalabis et al., 2005). The physiological relevance of this observation is not known, as placental *ABCB1* levels are lowest at late stages of pregnancy (Sun et al., 2006). Mina TH et al. suggested that this may partly explain the lower circulating cortisol in this cohort of severely obese pregnancy women (Mina et al., 2015). Global deficiency of *ABCB1* in rodents is thought to be associated with susceptibility to developing obesity (Foucaud-Vignault et al., 2011), and a polymorphism of *ABCB1* in humans associated with obesity (Ichihara et al., 2008). It is not known whether there is a genetic susceptibility of decreased *ABCB1* activity in this cohort of obese pregnant women, but this could be another mechanism underlying lower maternal cortisol.

Regulation of 11 β -HSD2 is tissue-specific, and may be altered in obese pregnancy. Placental 11 β -HSD2 activity has been shown to be altered with some of the hormones studied in this Thesis, or with other parameters known to be altered in obesity, such as inflammation. Progesterone and estrogen inhibit placental 11 β -HSD2 activity in term human placenta *in vitro* (Sun et al., 1998), and glucocorticoids down-regulate placental 11 β -HSD2 (Clarke et al., 2002, Kerzner et al., 2002). The lower circulating levels of glucocorticoids, estrogen and progesterone described in **Chapter 3** may limit the down-regulatory effect of these hormones on 11 β -HSD2 activity in obese pregnancy and may underlie increased fetal size via a reduction in fetal cortisol exposure. This may also be a potential mechanism contributing to the finding in **Chapter 4**, where increased BMI was associated with lower maternal cortisol, but not cord cortisol at delivery.

While not studied in this Thesis, pro-inflammatory cytokines, which are raised in obesity, also have an effect on 11 β -HSD2 regulation. Adipose tissue produces a significant amount of pro-inflammatory cytokines such as TNF α , and interleukin-6 (Galic et al., 2010). Further, in obese pregnancy, the relative increase in adipose

hypertrophy and hyperplasia leads to a dysregulated release of inflammatory markers (Avram et al., 2007, de Ferranti and Mozaffarian, 2008). Placentas from obese pregnant women have increased infiltration of macrophages, and increased expression of inflammatory markers (Challier et al., 2008). Chisaka et al., reported that in placental trophoblasts, TNF- α and interleukin-1 down-regulate 11 β -HSD2 mRNA expression and activity *in vitro* and suggested that TNF α and IL-1 may increase transplacental passage of cortisol from the maternal to fetal circulation by attenuating 11 β -HSD2 activity (Chisaka et al., 2005).

7.3 Strengths and Limitations

The strengths of this study are that a large sample size was used for characterizing the HPA axis hormones in **Chapters 3-4**, and initial studies examining possible mechanisms for these changes. **Chapter 5** dynamic studies of cortisol were performed. While the sample size was smaller for this, this is not unusual in more intensive dynamic testing studies. This study also involved both obese and lean non-pregnant controls, in order to study the relative contributions of pregnancy and obesity.

In **Chapters 4-6**, glucocorticoids were quantified using LC-MS/MS, which is the gold standard for measuring these analytes. Functional testing was also performed, with placental perfusion studies in **Chapter 6**.

Due to the time involved in developing the placental perfusion model and optimizing the assay for sample analysis, it was not possible to study the transport in a bigger sample of both lean and obese placentas for comparison.

The studies in this Thesis were limited by the absence of any longer-term follow-up studies. While significant progress has been made in understanding the effects of

maternal obesity on the HPA axis during pregnancy, longer-term programming effects are still being explored.

7.4 Future Directions

Targeted interventions for improving outcomes in pregnancies complicated by maternal obesity are limited. This is due to the combination of a lack of specific targets and also the unknown implications of interventions during pregnancy on the longer-term health of the offspring. Given the longer-term implications for offspring of obese, and the increasing support for the developmental origin of disease hypothesis, there is an urgent need for therapeutic targets and agents in obese pregnancy.

Two recent randomized controlled trials tested interventions aimed to reduce increased fetal size at birth in offspring of obese. The EMPOWaR study in Edinburgh tested whether administering metformin to obese pregnant women before any gestational diabetes was identified would affect offspring birthweight, but this study found no effect (Chiswick et al., 2015). The UPBEAT study tested a behavioural intervention in obese pregnant women, and concluded that lifestyle changes alone were insufficient to reduce the rates of gestational diabetes and large for gestational age infants (Poston et al., 2015).

The work in this Thesis suggests that glucocorticoids may be a possible new target for intervention, and provides a basis for a number of avenues that can now be further explored and a more complete understanding may be established. With further work, mechanisms by which maternal obesity alters the HPA axis could be ascertained, and potential therapeutic targets identified.

Further studies investigating impact of maternal obesity on HPA axis activity and

circulating cortisol levels could include analysis of the 11 β -HSD enzymes in central and peripheral adipose tissue, and studies testing the effect of both abdominal and generalized obesity on HPA axis activity in pregnancy. The influence of the HPA axis on other obese related pregnancy outcomes such as dysfunctional labour and prolonged pregnancy could be studied.

Placental cortisol metabolism studies could be extended by using the *ex vivo* placental perfusion model to investigate whether cortisol metabolism or transport is altered in obese compared with lean placentas. Infusion protocols could also be altered in order to study the transport from the fetal to the maternal compartment. Use of inhibitors of 11 β -HSD1, and the ABC transporters may give more detailed information about cortisol transport in the maternal-fetal barrier.

In vivo studies of placental cortisol metabolism in humans may be limited due the risk of potential harm to the developing fetus. Animal studies using a cortisol tracer could be used to study glucocorticoid transport between the maternal and fetal compartments during pregnancy. However, the major circulating glucocorticoid in rodents is corticosterone (as opposed to cortisol), so the translation of findings to human physiology may be limited.

As the importance of glucocorticoid pulsatility is increasingly recognized, future studies involving the delivery of glucocorticoids in a pulsatile fashion could be utilized to study the effects of glucocorticoid pulsatility on fetal or placental tissues (eg. *ex vivo* placental perfusion, or *in vivo* studies in adrenalectomised animal).

Longer-term offspring follow-up studies are needed to investigate whether altered glucocorticoid exposure *in utero* is associated with any change to the child's HPA axis sensitivity or diurnal rhythm. Studies are needed to ascertain the role of sleep in regulating the HPA axis, and whether maternal sleep problems could program a child's HPA axis or whether lower circulating cortisol could program a child's

diurnal rhythm.

While weight loss prior to pregnancy is encouraged, the effect of weight loss on the HPA axis is not well understood. Limited evidence from animal studies suggests that the HPA axis is activated in rats undergoing weight loss due to caloric restriction, but the equivalent weight loss by bariatric surgery prevents this (Grayson et al., 2014). In humans, calorie restriction has also been linked to increased total cortisol levels (Tomiyama et al., 2010). Further study in this area may inform best practice for optimizing pre-pregnancy weight loss and maternal cortisol levels.

7.5 Concluding Remarks

This Thesis found firstly that maternal obesity is associated with lower maternal cortisol levels and decreased HPA axis activity during pregnancy when compared to normal weight women. Secondly, these changes are maintained at the time of delivery in maternal, but not cord blood. Thirdly, altered glucocorticoid pulsatility may underlie these changes in obese pregnancy. Fourthly, the placenta does not appear to contribute significantly to maternal cortisol levels by the regeneration of cortisol from cortisone, and cortisol may not freely cross from the maternal to the fetal circulations.

Future studies could include more detailed analysis of the effects of altered glucocorticoid pulsatility in pregnancy, further investigation of placental cortisol metabolism and transport, and longer term offspring follow-up studies.

Altered glucocorticoid exposure *in utero* is likely to be one mode of programming a child's own health. A better understanding of HPA axis dysregulation in obese pregnancy could potentially inform us which high-risk pregnancies should be targeted to improve the health of the developing baby, and the safety of the mother.

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